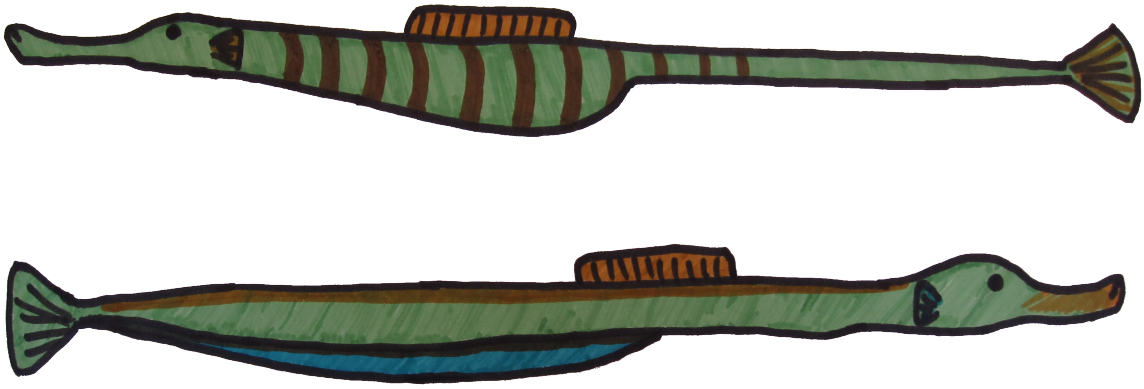
How salinity affects the pipefish-*Vibrio* interaction



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Abstract

Global change results in fast and severe alterations of environmental parameters. New environmental conditions that call for adaptations are created. For the Baltic Sea, a decrease in salinity is predicted and in particular shallow ocean habitats are highly influenced by the expected higher precipitation rates and temperatures due to the low water depth. Especially interactions between two species, such as host-parasite interactions, can be disrupted by environmental changes if the interacting species respond differently to the environmental changes. A resulting imbalance can possibly be deleterious for one of the species. Here, we investigated how the host-parasite interaction of pipefish (*Syngnathus typhle*), a small coastal marine fish, and bacteria of the genus *Vibrio* is influenced by changing salinities. Three salinity levels were used - highly brackish water (6 PSU), their habitual salinity (18 PSU), and an oceanic salinity (30 PSU). After acclimatisation to the new environment, the fish were injected with *Vibrio*. Pipefish up-regulated their immune defence upon salinity change, in particular if salinity was decreased. These environmentally stressed fish later lacked resources for an immune activation to fight the bacterial infection. This might decrease the survival rate and ultimately affect population size and distribution of *Syngnathus typhle*.

Zusammenfassung

Globaler Wandel hat schnelle und schwerwiegende Veränderungen von Umweltparametern zur Folge. Neue Umweltbedingungen werden geschaffen, die Adaptationen verlangen. Für die Ostsee wird eine Senkung des Salzgehaltes im Wasser vorhergesagt und vor allem untiefe Meeresstandorte werden stark von der erwarteten hohen Niederschlagsmenge und von hohen Temperaturen beeinflusst. Insbesondere Wechselwirkungen zwischen zwei Spezies, wie z.B. Wirt-Parasit-Beziehungen, können durch Umweltveränderungen gestört werden, wenn die Spezies verschieden auf die Veränderungen reagieren. Das resultierende Ungleichgewicht in der Wechselwirkung kann für eine der Arten schädliche Folgen haben. In diesem Projekt haben wir untersucht wie die Wirt-Parasit Beziehung zwischen der Grasnadel (*Syngnathus typhle*), einem kleinen Küstenfisch, und Bakterien von der Gattung *Vibrio* durch verändernde Salinitäten beeinflusst wird. Drei Salinitäten wurden für die Experimente benutzt - leicht salziges Wasser (6 PSU), die gewohnte Salinität der Fische (18 PSU), und ozeanische Salinität (30 PSU). Nach einer Angewöhnung an die neue Umgebung wurde den Fischen *Vibrio* Bakterien injiziert. Die Grasnadeln haben ihr Immunsystem als Antwort auf die Salinitätsveränderungen aktiviert, vor allem wenn der Salzgehalt des Wassers gesenkt wurde. Die durch die veränderten Umweltbedingungen beanspruchten Tiere hatten nicht mehr genug Ressourcen, um das Immunsystem nach einer bakteriellen Infektion zu aktivieren. Die Überlebensrate der Fische könnte dadurch beeinflusst werden, was schlussendlich die Populationsgrösse und Verbreitung von *Syngnathus typhle* beeinträchtigt.

Contents

1

Introduction

Changing environments pose a constant challenge for living organisms. Adaptations, i.e. the rearrangement (recombination or segregation) of genetic material or beneficial mutations, that result in a gain of fitness are essential for a species to persist under such circumstances (Bell & Collins, 2008). Various factors influence adaptations, in particular the speed and severity of change. While a high speed of environmental change makes beneficial mutations less likely to be established in a population, a severe change increases the selective pressure on the species (Bell & Collins, 2008). These two factors combined can result in constantly maladapted species which occasionally will go extinct. Especially species that interact with others are fragile and prone to be maladapted if the species respond differently to the environmental changes (Parmesan, 2006) and the interaction dynamics are disrupted. Such species interactions can occur in different ways: symbiotic (as in Mycorrhiza; Gerdemann, 1968), mutualistic (as in plants and its pollinators; Nilsson, 1988) or parasitic (as in host-parasite interactions; Sorci et al., 1997). Especially in host-parasite systems opposing reactions of the two interacting species to changing environmental conditions can lead to an imbalance that is possibly deleterious for one of the species. The host, which usually has a longer generation time, and the parasite, which has a short generation time and thus a higher potential for adaptation (Lenski et al., 1991), are in a constantly fluctuating interaction. In literature these dynamics are often referred to as the Red Queen (Van Valen, 1974). It describes the fluctuation of host and parasite genotypes, whereas rare alleles that result in higher fitness, i.e. increased likelihood to escape infection and higher virulence for the host and the parasite, respectively, have an advantage and are spread through the population by positive selection (Dybdahl & Lively, 1998). This pattern appears in both host and parasite, whereas there is a time lag between host and parasite genotype spreading (Figure 1.1). So, for example, the high virulence that results from a particular parasite genotype yields in spreading of the concerned allele. A host genotype that increases the likelihood of escaping infection is then beneficial and is also spread. The parasite loses fitness, because the dominant genotype is no longer virulent for the host and thus its prevalence is decreased. Hence, the beneficial aspect of the host's genotype is diminished and thus its prevalence is decreased as well. Then again, the cycle begins anew, as another rare allele has a beneficial

effect and is positively selected for. These dynamics require constant adaptation of both interacting species. If these dynamics are disrupted through environmental change, because one species is then better adapted than the other, this can result in extinction of one of the species.

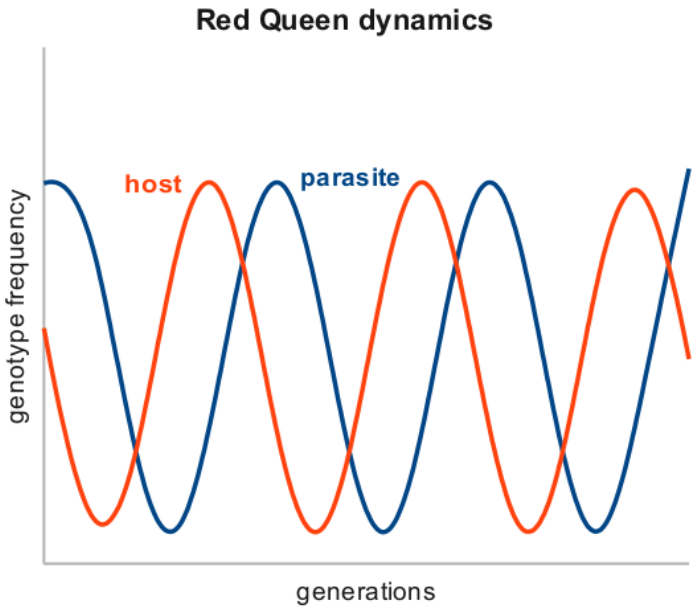


Figure 1.1: This figure shows the Red queen dynamics, i.e. the fluctuations of host and parasite genotypes, over time.

Global change is a widely known phenomenon, which results in drastic changes of environmental parameters (IPCC, 2007a). These changes affect a wide variety of environments all over the world. One of the most important ecosystems is the ocean, because it functions as a sink for carbon and other atmospheric gases (Siegenthaler & Sarmiento, 1993 and Sabine et al., 2004). Because this buffering function is climate dependent (high precipitation rates decrease ocean surface salinity and increase the stratification and thus decrease the sink strength of the ocean) the sink strength of the oceans is expected to decrease in the future (Sarmiento et al., 1998 and Cox et al., 2000). This means, that the environmental changes will be exacerbated due to the lack of buffering from the oceans. Currently, the temperature of the ocean is rising (IPCC, 2007a) and currents that are influenced by temperature gradients (e.g. Gulf Stream) are changing or slowing down (IPCC, 2007a). Global ocean salinity has been decreasing (Antonov et al., 2002) and is expected to drop during the next century due to increased intensity of precipitation (Karl & Trenberth, 2003) and ice cap melting (IPCC, 2007a). The massive ice caps are melting due to higher atmospheric temperatures, what, apart from freshening the ocean water, leads to an elevation of the sea level that is currently rising at the speed of 1.7 ± 0.3 mm per year (Church & White, 2006). Especially shallow water regions are highly affected by the mentioned changes, due to the proximity to the shores that are highly influenced by man (IPCC, 2007b) and the low water depth that results in higher fluctuations of

temperature and salinity due to insolation and rainfalls. Hence, organisms living in these environments are constantly challenged and are currently facing very drastic short- and long-term alterations as a consequence of global change.

Seagrass meadows are ubiquitous in the shallow coastal waters and play a significant role as ground stabilisation and as a nursery ground for fish and shellfish, whereas, based on abundance data, this role seems to be most important in the Northern hemisphere (Heck Jr. et al., 2003). A trophic cascade that reaches from microalgae at the lower end to predatory fish at the upper end enables this ecosystem to abide (Moksnes et al., 2008). If one organism from this cascade is removed, the dynamics are brought out of balance and microalgae abundance is ultimately affected, which in turn affects the seagrass popoulation. Thus, a disruption of this cascade would indirectly result in a decline of juveniles of fish that are partially of commercial importance (Heck Jr. et al., 2003). Environmental change can thus endanger one of the human's important food sources.

For the Baltic Sea the results of global change are predicted to be especially pronounced in higher deep and surface water temperatures (MacKenzie et al., 2007) and more frequent extreme climate events, such as heatwaves (Frich et al., 2002). Also, changes in salinity are particularly an issue in the Baltic Sea, as it is basically isolated from other marine systems (Johannesson & André, 2006) and thus is predicted to decrease in salinity due to high intensity of precipitation and river water run-off. Decreased salinity has been shown to result in a decline of copepod biomass proportion in the Baltic Sea (Vuorinen et al., 1998) what induced a starvation of fish populations, in particular herring, (Flinkman et al., 1998) and resulted in a decreased weight of the commercially caught fish.

Experimental evidence on how parameters that are expected to be altered by global change affect species interactions is scarce - only few researches have been done (e.g. Roth et al., 2010 and Landis et al., in press). Therefore, we here investigated experimentally how the host-parasite interaction of a small coastal marine fish and bacteria of the genus *Vibrio* is influenced by changing salinities. Decreasing salinity is expected to increase the virulence of *Vibrio spp.* (Kelly, 1982) and thus to decrease host fitness due to higher severity of *Vibrio* infections. In the experiments presented here, we tested for short-term adaptation to changing salinities in a realistic scenario: Pipefish of the species *Syngnathus typhle* were slowly adapted to changing salinities and artificially infected with a combination of allopatric phylotypes of *Vibrio spp.* Upon infection the activation of the immune defence was measured as a proxy for host resistance and an estimation of its physiological status. Measurements were performed using head kidney and blood cells, since the previous is the place where the immune cells are synthesised and the latter is the vector that transports the needed cells to the periphery. Furthermore, the expression of a set of innate and adaptive immune genes was assessed using the gills of the fish, because they are in direct contact with the surrounding water and thus play a major role as a first barrier of the immune defence. The mentioned scenario was simulated not only for adult pipefish that had been caught in the wild, but also for juvenile pipefish that were bred in the laboratory. This allowed us to investigate the consistency of immune parameters between young and adult pipefish and to disentangle effects of a potential adaptive immune memory due to previous *Vibrio* exposure in the wild (for the adults) and direct

immune defence upon first exposure (for the juveniles). The results of this study provide to the best of our knowledge the first data that gives an insight in how a host-parasite system is influenced by changes in salinity and can give important implications on how future scenarios of global change may affect host-parasite interactions.

2

Materials and Methods

2.1 Model organisms

Broadnosed pipefish (Figure 2.1), (*Syngnathus typhle*), are native to shallow eelgrass (*Zostera marina*) meadows of the European coastline (Ahnesjö, 1994). They are sex-role reversed, such that the males invest more in the offspring and are the choosier sex (Vincent et al., 1992). The females perform a courtship dance (Berglund, 1993) by displaying their striped ornaments (Berglund & Rosenqvist, 2001), which also function as an intimidating signal for the competitive individuals of the same sex (Berglund & Rosenqvist, 2009). When a male has chosen a female for copulation, the eggs are transferred from the female into the male's brood pouch by a small ovipositor. The eggs are then fertilised in this brood pouch and are provided with oxygen and nutrients through a placenta-like structure (Berglund et al., 1988). After about 30 days of carriage, the brood pouch is opened and the offspring was born alive (Berglund & Rosenqvist, 1993). The juveniles are then immediately independent from the male parent, which usually goes to find a new mating partner. Multiple matings for both sexes are common for *S. typhle*.
This sex-role reversed fish is a dominant and very important species in the seagrass environment, because they have a top-down influence on the trophic chain. They directly regulate the amount of invertebrates such as isopods by feeding on them, which further feed on the microalgae on the seagrass. They thus help to maintain the trophic cascade that enables the survival of the seagrass meadows (Moksnes et al., 2008). The pipefish are very bad swimmers, but their body is designed to be very well camouflaged in the seagrass (Vincent et al., 1994). Because of these characteristics and the shallow feature of their habitats, they are exposed to significant changes of environmental parameters and cannot avoid them by moving to a less affected part of the sea.

Syngnathidae serve as a host for *Vibrio spp.* (Alcaide et al., 2001), one of the most important pathogens in the ocean. It is a motile, rod-shaped, gram-negative bacterium which is ubiquitous in the marine environments (Thompson et al., 2004). *Vibrio* is a pathogen of a wide variety of fish, in addition to the broadnosed pipefish, and leads to a disease that is called vibriosis. This disease, at a later stage, results in external and internal haemorrhages (Alcaide et al., 2001) and is potentially lethal.

(a) Pipefish in an aquarium with artificial seagrass.

(b) Close-up picture of the typical pipefish head.

Figure 2.1: Broadnosed pipefish, *Syngnathus typhle*.

2.2 Fish catching and husbandry

The fish were caught by pulling handnets through the seagrass meadow while snorkelling in water depth of 1-3 meters. All fish for the Experiment 1 were caught at the beginning of June 2011 in Strande, Germany (N 54°43.39'; E 10°16.93'). Until the start of the experimental phase, the fish were kept in 200 liter barrels containing filtered Baltic Sea water, oxygen stones and artificial segrass. They were fed a mixture of frozen and live mysid shrimps twice a day. The juvenile fish for Experiment 2 were all born in the lab, the parental fish were caught in Strande, Germany, and were only used for breeding. For this, fish of both sexes were kept in big barrels and allowed to mate randomly. As soon as the young fish were born, they were separated from the parents and kept in aquaria with a volume of 20 liters. They were fed with live artemia nauplii and wild caught copepods. The light cycle in the husbandry room was adjusted so that the fish experienced a typical summer cycle with light from 5am to 10pm.

2.3 Experiment 1 - Mature fish

During the experimental phase the fish were kept in 20 liter buckets with an oxygen stone and an artificial seagrass in 3 different salinities. We used 21 buckets, i.e. seven per salinity, each containing nine fish and arranged them randomly. Baltic Sea water with a mean salinity of about 17 PSU was used during the whole experiment. Lower salinities were achieved by mixing with tap water and higher salinities were produced by addition of nitrate-free synthetic sea salt (Instant Ocean®). To all water mixtures a conditioner

was added to remove the chloride from the tap water (Stress Coat®from API Aquarium Pharamceuticals) . Again, the fish were fed twice a day and half of the water in the buckets was exchanged every second day.
For the first day of the experiment, all fish were kept in water with 18 PSU (mean salinity at their origin), then the salinities were changed during 3 days in steps of 4 PSU per day, until the final experimental salinities were reached: 6, 18 and 30 PSU. On the last day of the salinity acclimatisation phase, i.e. on day four of the experiment, the injections were done. For these injections we have chosen *Vibrio* strains, according to a phylogeny of *Vibrio* related to pipefish of the species *S. typhle* (Roth et al., in review), so that potential previous exposure of the fish to *Vibrio* could not confound our data, i.e. the injected bacteria were expected to predominate the fish's earlier encounters with *Vibrio*. A mix of eight allopatric *Vibrio* strains was prepared: D11K1, I11E3, I2K1, D1K1, SH54, D1K3, D1E3, D12K2, where the first letter is according to the origin - Italy (I), Denmark (D) or Sweden(S) - and the second letter describes where the bacteria was extracted from - gills (K) or eggs (E) of the pipefish or from the surrounding water (H). The bacteria were taken from -80°C glycerol stocks and were grown on a petri dish filled with *Vibrio* selective (Thiosulfate Citrate Bile Salts Sucrose (TCBS)) agar at 25°C over night. The following day, a single colony was picked and suspended in 4ml liquid medium (Medium 1) and grown in an over night culture at 25°C. The next morning, the solution was centrifuged at 2500rpm for ten minutes. The supernatant was then removed and the bacteria were dissolved in 1ml of phosphate buffered saline (PBS). All strains were subsequently mixed, centrifuged again (2500rpm for ten minutes) and dissolved in 5ml of PBS, yielding a concentration of 10^7 bacteria cells per ml. 36 fish per salinity, i.e. four buckets, were injected with 10μl of the allopatric bacteria mix. The remaining 27 fish (three buckets) per salinity were injected with the same volume of PBS, as a control for the injection procedure. The injection itself was performed with a 0.3 mm needle. The needle was inserted angularly, facing the head, into the abdominal cavity of the fish, i.e. on the ventral side. The needle was kept shallow in order to not inflict any damage to the intestine. The very low mortality rate (only one infected fish from 6 PSU died in the following days) showed that the procedure itself did not harm the fish.
The first immune measurements were taken one day after infection. For that, 12 fish per treatment (6 treatments in total) were randomly picked and had to be sacrificed. They were killed, the weight and length were measured and the sex and pregnancy status (in case of the males) were noted. Then the tail was cut off and the blood was pressed out into RPMI-1640 cell medium (from SIGMA®). Also, the head kidney (in the broadnosed pipefish this is equivalent to the upper third of the kidney strains) was removed, squashed through a cell sieve and the obtained cells were diluted in the same cell medium as the blood. Finally, from the head, the gills were taken and put into an RNA stabilisation reagent (RNA*later*^{TM}) for subsequent gene expression measurements. The exact same procedures were done for the second measurement point, i.e. eight days after infection. Since 12 replicates were used per treatment and we had three salinities, two infection states (control and *Vibrio*) and two timepoints of dissection, we used a total of 144 pipefish for this experiment.

2.4 Experiment 2 - Juveniles

When the juvenile fish, that had been obtained from random mating of fish caught in Strande, had an age of about 4 weeks, the experiment was started. As in Experiment 1, the fish were acclimatised to the three differnet salinities during 3 days. We used 21 buckets, with five liters water each, in a randomised design. Eight to nine fish were held in one bucket and there was an oxygen stone in each, but no seagrass. The juveniles were fed a big amount of live artemia nauplii once a day and the water was exchanged every second day. This experiment was conducted at the Research Station Ar on Gotland, Sweden. Thus more artificial sea salt had to be used to generate the experimental salinity conditions, because the ambient salinity was lower (around 7 PSU) than in Kiel, Germany. The infections were conducted on the fourth day of the experiment. This time only one allopatric strain was used, I9K1. The concentration of 10^7 bacteria cells per ml was achieved the same way as for Experiment 1. Again, a 0.3 mm needle was used and the fish was pricked on the ventral side. This time, however, no liquid was injected, but the needle was dipped either into the *Vibrio* solution or into PBS before the sting, as is done for other very small organisms such as beetles (Roth & Kurtz, 2009). Even applying this minimal invasive procedure, the deathrate after the infections was very high in all three salinities, especially in 30 PSU where all fish died, independently of the fact if the fish was challenged with *Vibrio* or not. During the first three days after infection, 8 control fish and 11 infected fish in 6 PSU, 8 control and 6 infected fish in 18 PSU, and 14 control and 20 infected fish in 30 PSU had died.
One day after the immune challenge, due to the high deathrate, only five to seven fish per treatment were sacrificed, in order to be able to perform a second measurement with at least five replicates. The second measurement had to be done five days after infection, because in one treatment only five fish were left by then and the probability that they all survive until day eight was very low. The length of the fish was measured before their head was cut off to kill them. The head and body of the fish was put into 300μl of RNA*later*^{TM} for subsequent gene expression analysis. We here concentrated on quantification of gene expression, as the size of the fish did not allow direct measurements of immune parameters.

2.5 Immune assays

The cellular immune assays were all conducted with the freshly isolated cells, at the same days of the dissections. For this, a fluorsecent-assisted cell sorting (FACSCalibur by BD Biosciences) machine and the CellQuest Pro Software (also BD) were used. The sorting procedure was based on a programme that defined immune cell characteristics, such as the size and the complexity (monocytes are much bigger and more granulous than lymphocytes) (Roth et al., 2011). Two counting processes were performed. First, the FACS counted all live cells, single cells, monocytes (cells from the innate immune system) and lymphocytes (cells from the adaptive immune system) to measure the activation of the innate and aptive immune system, respectively. For this, the number of monocytes and lymphocytes was divided by the number of live cells that were counted. The resulting proportion of immune cells gives an estimate of the immune system activation. In a second counting procedure the cell cycle stage of the lymphocytes was determined to check

the proliferation activity of the adaptive immune cells. We here differentiated resting stage (G01), activated cells with doubled chromosomes (S), or cells in the stage of myosis and cell division (G2M) (Figure 2.2).

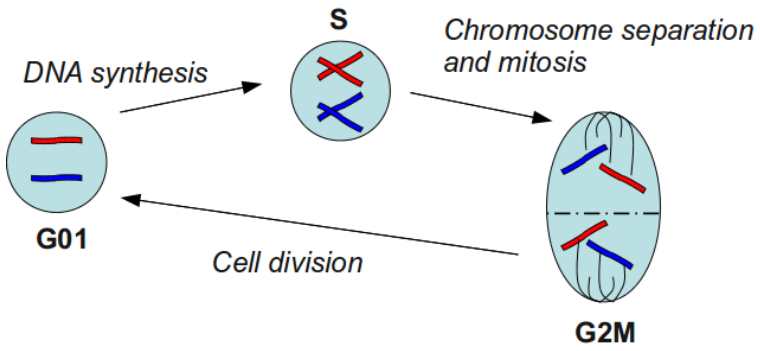


Figure 2.2: This figure shows the three different cell cycle stages. When a resting cell (G01 phase) is activated, it undergoes DNA synthesis and chromosome duplication (S phase). This is followed by a mitosis (G2M phase) and cell division, which yields two diploid cells.

The obtained blood and head kidney cells were cleaned and concentrated by centrifugation followed by re-uptake with 450μl Megacell. Then the cells were randomly pipetted to the plates in two combinations for the two different counting procedures. First, 50μl of cells were mixed with 150μl of FACS flow (the sheath fluid of the FACS), 70μl propidium iodide (PI, stains the DNA of dead cells) and 30μl of a known concentration of beads (as a reference to count cells). The second combination consisted of 50μl of cells with 200μl ethanol (to disrupt the cell membranes to make the DNA available for PI staining), 70μl PI and 25μl beads. During the whole procedure the cells were kept on ice.

To determine the activity of the innate immune cells (monocytes), a respiratory burst assay was performed that gives an estimate of the phagocytotic activitiy of the cells. This was only done for the blood cells, because not enough monocytes were counted in the head kidney in order to achieve the needed concentration of cells. For this the remaining cells from the dissection were diluted to achieve a concentration of $1.25\text{x}10^6$ cells per ml, based on the counting results from the FACS. 80μl of this cell solution was mixed with 80μl of MegaCell solution, 20μl lucigenin (a luminescent chemical that binds to oxygen radicals) and 20 μl zymosan (a cell wall compound from yeast). The activated monocytes, such as macrophages, start to phagocytose the yeast compounds (zymosan). During this process oxygen radicals are released, which activate the lucigenin. This lucigenin is then trasformed to luciferase and energy in the form of luminescence is emitted. A machine that measures this luminescence (Tecan infinite M200) was used. The detected intensity of luminescence reflects the rate of phagocytosis and thus the activity of one parameter of the innate immune system.

2.6 Gene expression assay

The gene expression assay was done by means of quantitative real-time reverse transcription PCR (QPCR). This technique is used to determine the number of transcripts of a particular gene present in a template. A fluorescent dye is used that explicitly binds to double-stranded DNA. After every PCR cycle the fluorescence is measured. Based on the resulting amplification curve, the original amount of cDNA and accordingly RNA can be calculated.
From Experiment 1 the RNA from the gills of the first dissection date was extracted. From Experiment 2 RNA from whole juvenile fish of both dissection dates was extracted and used for quantification of gene expression. Due to time constraints, only from five replicates of both experiments gene expression was quantified. The RNA was extracted using the InviTrap®Spin Tissue RNA Mini Kit from Invitek. RNA was immediately frozen at -80°C. The RNA extraction yields were measured using a spectrophotometer (NanoDrop®ND-1000 from peQLab). For the reverse transcription (RT) of the RNA, the QuatiTect®Reverse Transcription Kit from Qiagen was used, because it encluded a gDNA wipeout buffer. This buffer was needed to ensure that after the RT only cDNA was present, and no gDNA (see Appendix). The amount of RNA was chosen such that the resulting cDNA, after a 1:5 dilution to increase the volume, had a concentration of $0.005\mu g/\mu l$. The cDNA was stored at -20°C.
Ten immune genes were selected from the expressed sequence tag (EST) library of *S. typhle* immune genes (library by O. Roth & D. Haase): complement component 3 (C3), coagulation factor II receptor-like 1 (cf), heat-shock 60kDa protein 1 (hsp1), interleukin 10 (IL10), lymphocyte antigen 75 (LA), toll-like receptor 5 (TLR), kinesin family member 13b (kin), natural resistance-associated macrophage protein (nramp), granulocyte colony-stimulating factor precursor (grcsf) and tumor necrosis alpha-induced protein 8-like 2 (tnf). Furthermore, the housekeeping gene ubiquitin (ubi) was included in the measurements and served as an internal control for the calculations of the relative gene expressions. The functions of the single genes are explained in Table 7.2 in the Appendix.
Every well of the gene expression assay contained $0.2\mu l$ of forward and reverse primers ($50pmol/\mu l$), $5.6\mu l$ of Milli-Q water (from Millipore), $10\mu l$ of Fast SYBR®Green Master Mix (Applied Biosystems) and $4\mu l$ of template. Each gene was measured in triplicates for every individual. Also, a run was done for every template with the non-reverse transcribed RNA after the gDNA wipeout step with the primers for ubiquitin to make sure that all gDNA was denatured. For all master mixes a non-template control (NTC, $16\mu l$ master mix + $4\mu l$ water) was conducted to check for contaminations. The triplicates and negative controls were randomly distributed on the plates in order to randomise a possibly occurring plate effect.
The QPCR protocol was done as follows: the samples were heated up to 95°C for 20s for complete denaturation and then 40 cycles of 95°C for 3s and 60°C for 30s were performed. This thermocycling procedure was followed by a dissociation phase, where the samples were slowly heated from 65°C to 90°C. The resulting melting curve (Figure 2.3) was used to check that the PCR reaction only yielded a single product. The StepOne Plus Real-Time PCR System (Applied Biosystems) machine measured the number of cycles (C_T) that was needed for each sample to reach a linear character in the amplification curve (Figure 2.4(a)). The C_T threshold, i.e. where the fluorescence of the sample clearly

outranges the background fluorescence, was manually set to 0.5 for all genes (Figure 2.4(b)). Samples that were not amplified or samples of which the RNA negative control was amplified were excluded from the analyses. There was some amplification in the RNA negative controls, where the C_T was at least five values higher than the amplifications of the cDNA samples. However, since the PCR process leads to an exponential production of cDNA, the contribution of the probably little amount of remaining gDNA to the C_T value can be ignored. For all measurements, the standard deviations (SD) and means were calculated. Further, the coefficient of variance (CV) was determined using Formula 2.1a. For the triplets where CV>0.04 (Bookout & Mangelsdorf, 2003), an additional gene expression measurement should be done in order to identify outliers. Due to time constraints we just left out the few measurements from further analyses, where the CV was higher than 4%. The relative gene expression of every gene, $-\Delta C_T$, was calculated using Formula 2.1b. With these values all plots and all statistical analyses were conducted. Furthermore, a value for $-\Delta\Delta C_T$ (Formula 2.1c) was calculated for every gene in every individual. Applying the Formula 2.1d to these values yielded the relative quantity (RQ) of every gene, i.e. the amount of the gene under treatment conditions (here *Vibrio* infection) is RQ times the level of the gene in the control treatment (VanGuilder et al., 2008).

$$CV = SD_{C_T}/C_{Tmean} \tag{2.1a}$$

$$-\Delta C_T = C_{T(ubi)mean} - C_T \tag{2.1b}$$

$$-\Delta\Delta C_T = -\Delta C_{T(vibrio)} - (-\Delta C_{T(control)}) \tag{2.1c}$$

$$RQ = 2^{-\Delta\Delta C_T} \tag{2.1d}$$

2.7 Statistics

Two programmes were used for the statistical analyses. R version 2.11.1 was used for all Analyses of Variance (ANOVAs) and multivariate analyses of variance (MANOVAs), and Primer v6 was used for Similarity Percentage (SIMPER) tests. The data were tested for normality using the Shapiro-Wilk test. If the data were not normally distributed, a transformation was done before conducting the statistical tests. In all ANOVAs and MANOVAs the salinity, infection status (control or *Vibrio*) and timepoint of dissection were included as fixed factors. All significant ANOVAs and MANOVAs were followed by a post-hoc Tukey's "Honestly significant difference" (TukeyHSD) test. For the SIMPER tests that were conducted with the gene expression data, all negative values had to be eliminated from the data set, because a fourth root transformation was done to enable the comparison of very high and very low values (Bergmann et al., 2010). The negative $-\Delta C_T$ values were eliminated by addition of a constant to all the values of the concerned gene. Also, the SIMPER test cannot deal with missing values, which meant that all eliminiated values (due to CV or negative control) were replaced by the mean expression value of the affected gene (detailed information about the number of replicates for every gene can be found in the Results). This enabled the testing without having to eliminate the whole data of several samples.

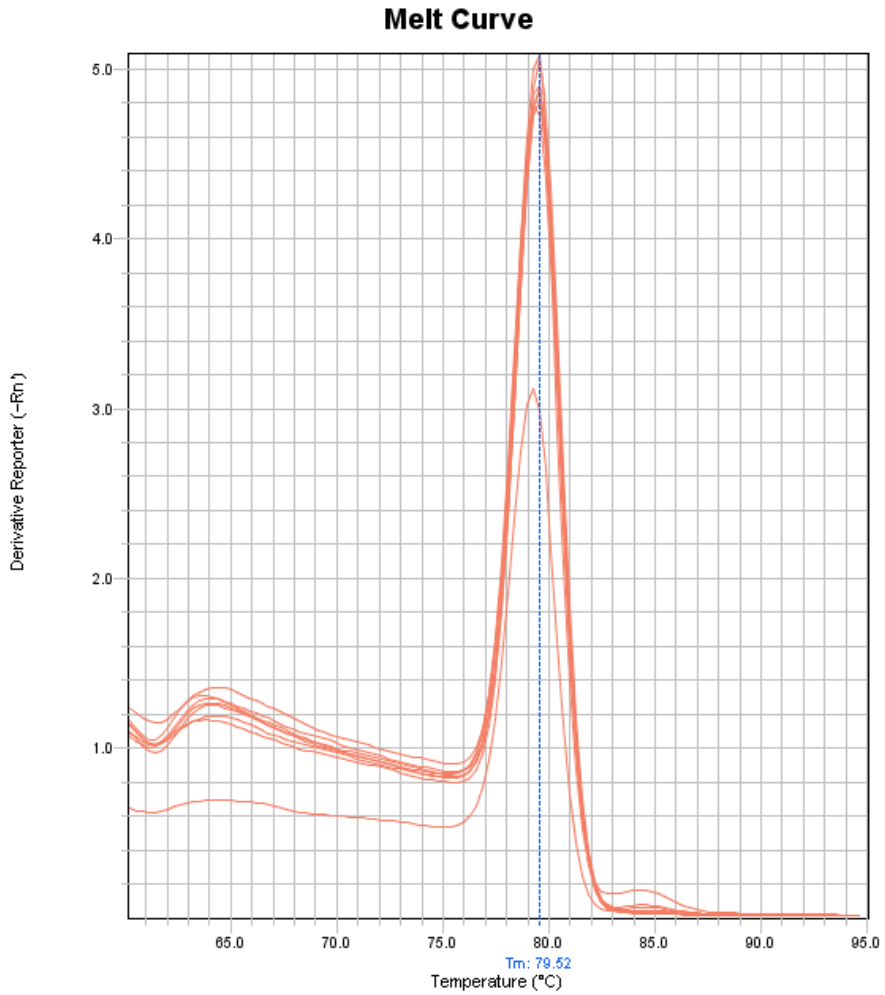
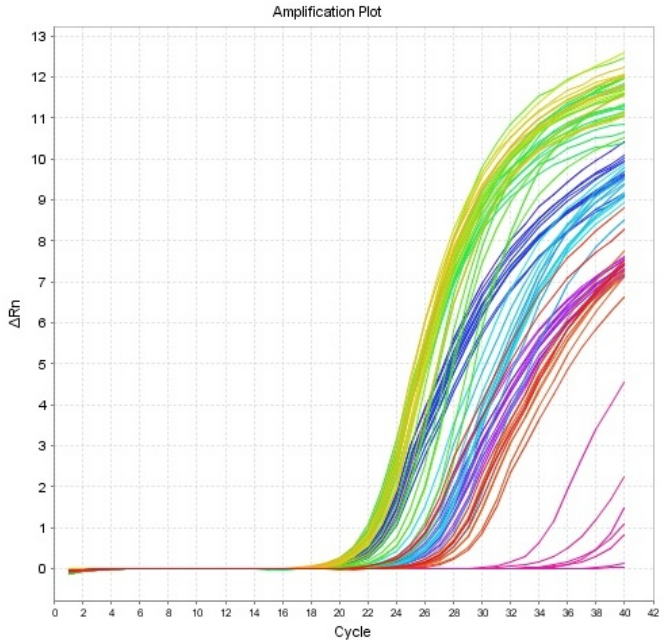
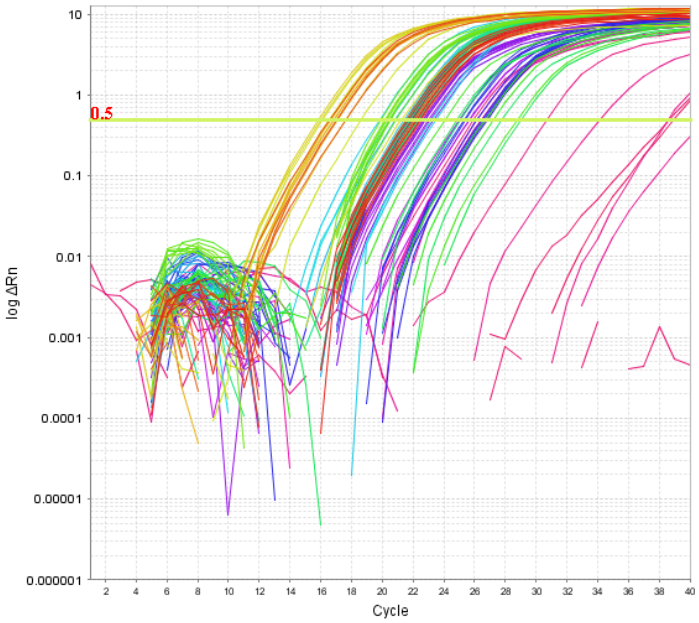


Figure 2.3: Melt curve of the same run as the amplification plots in Figure 2.4(a). Again, the colours correspond to the different genes on the plate. All reactions only yielded one product with a T_m around 80°C. The occasional peaks under 70°C are primer-dimers that can be ignored.

For analysis of the lymphocyte activity of the fish that were dissected eight days after infection, the results of plate three were excluded, because the mean proportion of cells in the G01 phase over all treatments on plate three was 0.24, whereas the mean for plates one and two were around 0.7 (data not shown). This enormous difference lead us to the assumption that the counting procedure had not functioned correctly, since it was known to us that the FACS machine sporadically had problems with the cell sorting.



(a) Linear amplification plot. The higher the cDNA concentration in a sample, the earlier the curve will reach the linear phase. The pink curves on the right of the plot are negative controls.



(b) Logarithmic amplification plot. Here, the manually set threshold of 0.5 is shown. The threshold was set so that it is in the log linear phase of the amlification curve and definitely above the background fluorescence for every gene.

Figure 2.4: Typical amplification plots from the $StepOne^{TM}$ Plus Software v2.0 (Applied Biosystems), displayed as a linear and a logarithmic plot of the same run. The x-axis shows the number of PCR cycles and the y-axis shows the intensity of fluorescence, ΔRn. The lines are coloured according to the different genes measured on this plate, so all curves with the same colour represent the measurements of the same gene but from several individuals.

3

Results

All shown figures are boxplots that depict the median, the upper and lower quartile, the sample observation range and the probable outliers.
In this section only the p-values of the statistical tests are shown. For more detailed statistical results see Appendix.

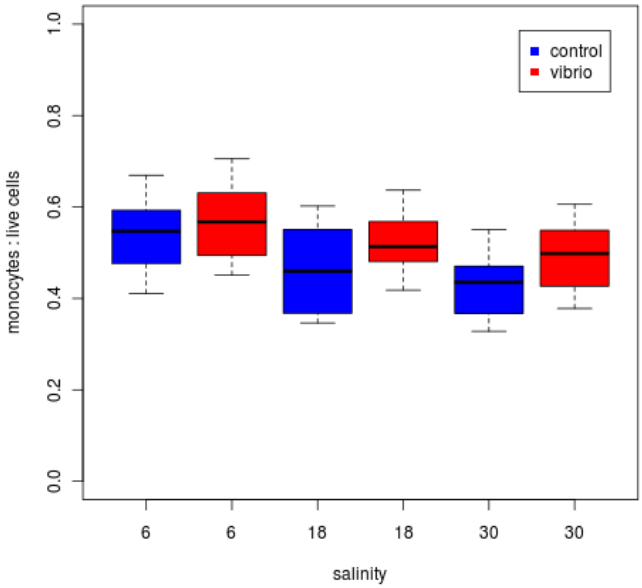
3.1 Experiment 1 - Mature fish

Cell count

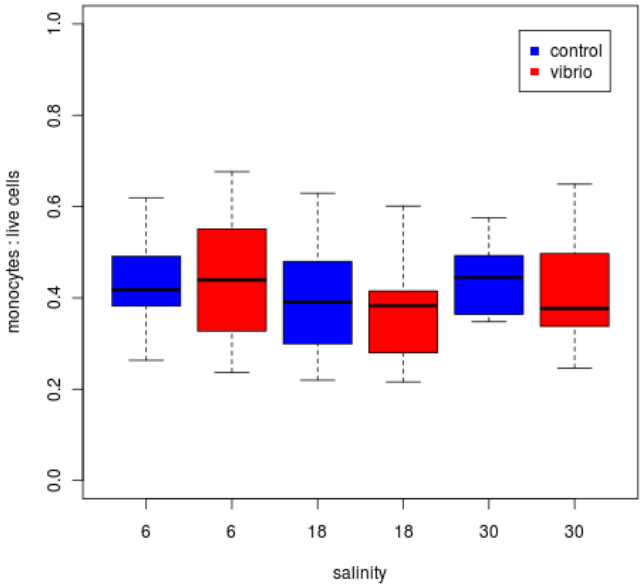
Figure 3.1 shows the amount of monocytes in the head kidney of the fish one and eight days after infection. The results of the according ANOVA show that animals from the low salinity treatment had the highest monocyte count (Table 3.1). No significant effect of *Vibrio* infection was found. In the plots one can clearly see that fish from the salinity 6 have the highest proportion of monocytes both in the control and *Vibrio* treatment. The fish from 18 and 30 PSU have more monocytes when their immune system is challenged with *Vibrio*, what suggests that the activation of their innate immune system has taken place. The fish from salinity 6, however, fail to up-regulate their innate immune system further, when they are challenged with the infection. In the blood (Figure 3.2) also salinity and timepoint are significant (Table 3.1). Here, the salinity effect is due to the intermediate salinity 18. The amount of monocytes in the intermediate salinity is overall lower than in the other two salinity treatments.
The plots for the proportion of lymphocytes (Figures 7.1 and 7.2) can be found in the Appendix. They are not further discussed, since the amount of lymphocytes negatively correlates with the amount of monocytes, because the amount of lymphocytes plus monocytes proportionally corresponds to the number of live cells counted by the FACS.

Lymphocyte activity

Figure 3.3 shows the proportion of lymphocytes in the head kidney that are in the G2M (mitosis and cell division) phase one and eight days after infection. The results from

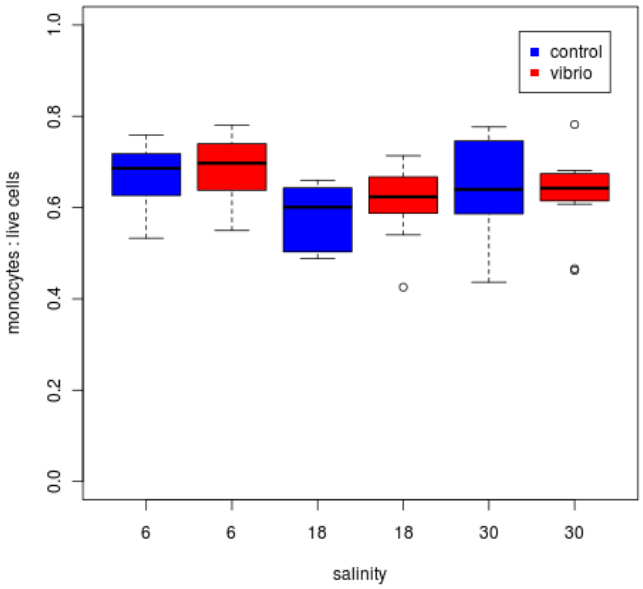


(a) Proportion of monocytes in the head kidney of the fish one day after infection.

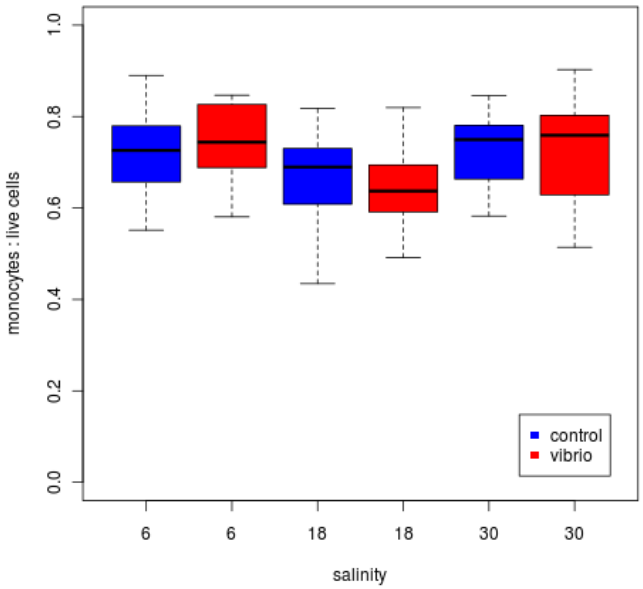


(b) Proportion of monocytes in the head kidney of the fish eight days after infection.

Figure 3.1: The proportion of monocytes, i.e. the number of monocytes divided by the number of total live cells counted, in the head kidney of the fish from the different treatments one and eight days after infection is shown. The blue bars represent fish from the control treatment and the red bars show the data for the fish that were infected with *Vibrio*.



(a) Proportion of monocytes in the blood of the fish one day after infection.



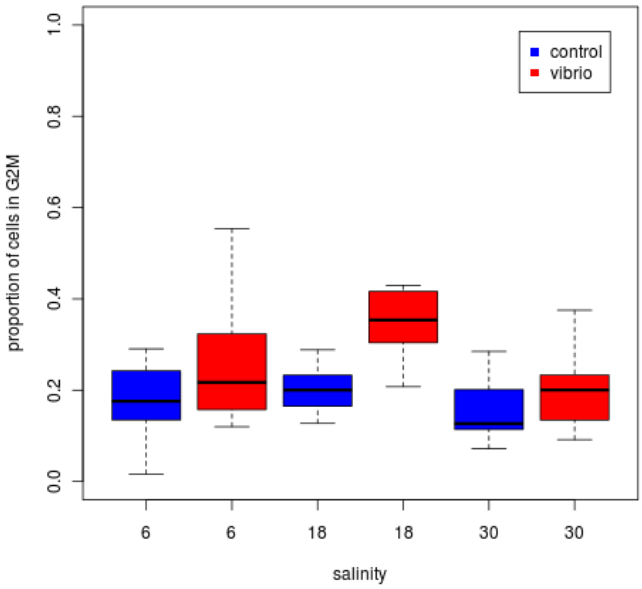
(b) Proportion of monocytes in the blood of the fish eight days after infection.

Figure 3.2: The proportion of monocytes, i.e. the number of monocytes divided by the number of total live cells counted, in the blood of the fish from the different treatments one and eight days after infection is shown. The blue bars represent fish from the control treatment and the red bars show the data for the fish that were infected with *Vibrio*.

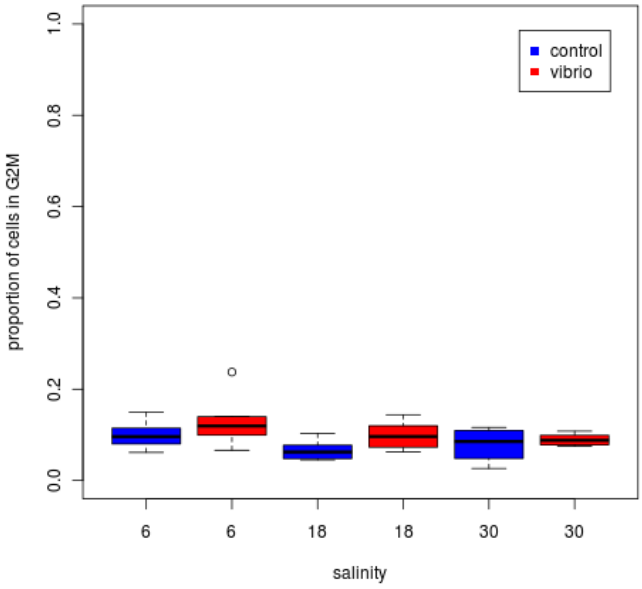
Table 3.1: The results of the statistical tests for the proportion of monocytes in the head kidney and blood of the fish are shown. Timepoint 1 is defined as one day after infection, whereas timepoint 2 represents the data from eight days after infection. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.3 in the Appendix.

parameter	***head kidney*** **p-value**	***blood*** **p-value**
ANOVAs		
salinity	**0.0110**	**0.0001**
vibrio	0.3090	0.7168
timepoint	**1.06E-06**	**1.63E-05**
salinity*vibrio	0.9818	0.6776
salinity*timepoint	0.0679	0.6301
vibrio*timpeoint	**0.0421**	0.6308
salinity*vibrio*timepoint	0.4968	0.6145
TukeyHSD		
18PSU-6PSU	**0.0174**	**8.23E-05**
30PSU-6PSU	**0.0354**	0.3422
30PSU-18PSU	0.9618	**0.0100**
timepoint1 - timepoint2	**1.1E-06**	**1.63E-05**
vibrio:tp1 - control:tp1	0.1284	
control:tp2 - control:tp1	0.1318	
vibrio:tp2 - control:tp1	**0.0235**	
control:tp2 - vibrio:tp1	**1.44E-04**	
vibrio:tp2 - vibrio:tp1	**8.1E-06**	
vibrio:tp2 - control:tp2	0.8989	

the statistics are shown in Table 3.2. Significant results were found for *Vibrio*, timepoint and for the salinity*timepoint interaction. The post-hoc test for the interaction shows that for the first timepoint salinity 18 is different from the other two, but for the second timepoint there are no significant salinity effects. In the blood, salinity and *Vibrio* have a significant effect on the activity of the lymphocytes. The salinity effect is due to the difference between salinity 6 and 18. The high salinity seems to result in a lymphocyte activity that is between the low and intermediate salinity. *Vibrio* infection leads to a higher lymphocyte activity.
The plots of the cells in the resting (G01) and DNA synthesis (S) phase for the head kidney and blood are found in the Appendix in Figures 7.3 and 7.4 , respectively.



(a) Proportion of lymphocytes in the head kidney that are in the mitosis and cell division stage (G2M) one day after infection.

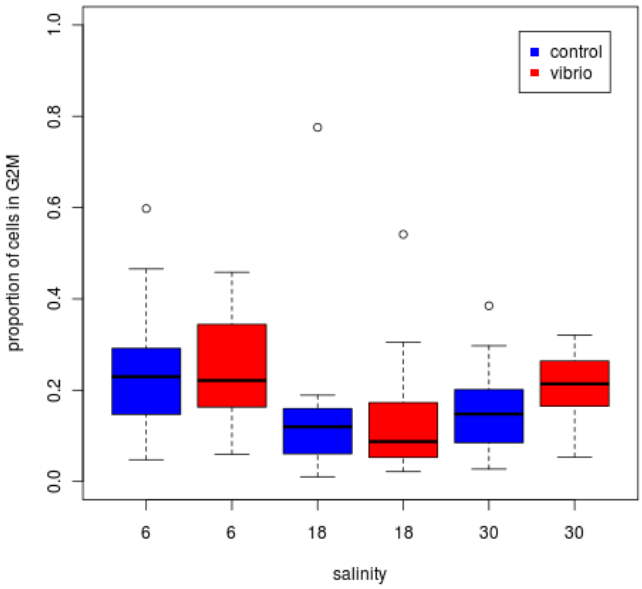


(b) Proportion of lymphocytes in the head kidney that are in the mitosis and cell division stage (G2M) eigt days after infection.

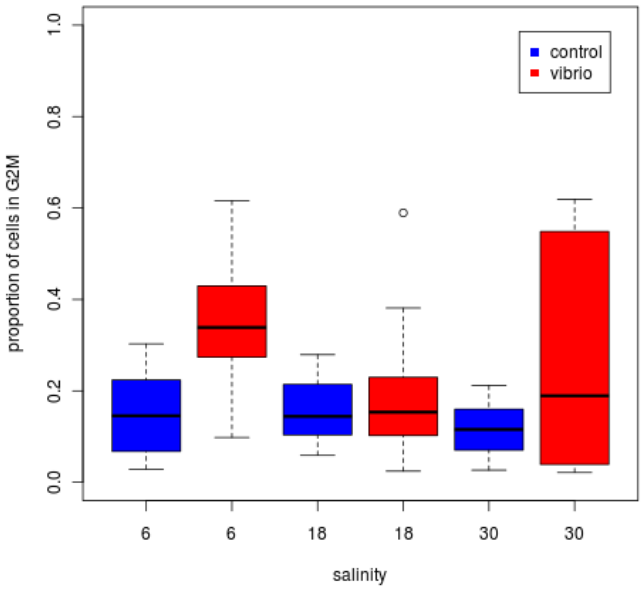
Figure 3.3: The proportion of lymphocytes, i.e. cells from the adaptive immune system, in the head kidney that are in the mitosis and cell division (G2M) stage, one and eight days after infection is shown. The blue bars again represent the fish from the control treatments, whereas the red bars stand for the fish that were infected with *Vibrio*.

Table 3.2: The results of the statistical tests for the proportion of lymphocytes in stage G2M in the head kidney and blood of the fish are shown. Timepoint 1 is defined as one day after infection, whereas timepoint 2 represents the data from eight days after infection. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.4 in the Appendix.

parameter	***head kidney*** **p-value**	***blood*** **p-value**
ANOVAs		
salinity	0.0632	**0.0264**
vibrio	**4.1E-05**	**0.0345**
timepoint	**<2.2E-16**	0.8921
salinity*vibrio	0.7116	0.5180
salinity*timepoint	**0.0017**	0.2514
vibrio*timepoint	0.4099	0.1584
salinity*vibrio*timepoint	0.9311	0.4555
TukeyHSD		
18PSU-6PSU		**0.0206**
30PSU-6PSU		0.2453
30PSU-18PSU		0.5786
vibrio-control	**4.75E-05**	**0.0359**
timepoint1 - timepoint2	**0**	
18:tp1 - 6:tp1	**0.0361**	
30:tp1 - 6:tp1	0.8732	
6:tp2 - 6:tp1	**9.1E-04**	
18:tp2 - 6:tp1	**1E-07**	
30:tp2 - 6:tp1	**2.6E-06**	
30:tp1 - 18:tp1	**8.2E-04**	
6:tp2 - 18:tp1	**0**	
18:tp2 - 18:tp1	**0**	
30:tp2 - 18:tp1	**0**	
6:tp2 - 30:tp1	**0.0253**	
18:tp2 - 30:tp1	**1.03E-05**	
30:tp2 - 30:tp1	**1.09E-04**	
18:tp2 -6:tp2	0.3562	
30:tp2 -6:tp2	0.4499	
30:tp2 - 18:tp2	1.0	



(a) Proportion of lymphocytes in the blood that are in the mitosis and cell division stage (G2M) one day after infection.

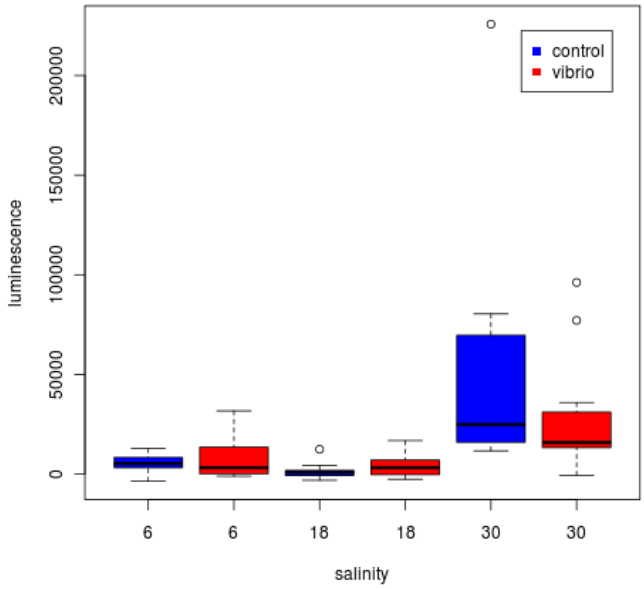


(b) Proportion of lymphocytes in the blood that are in the mitosis and cell division stage (G2M) eight days after infection.

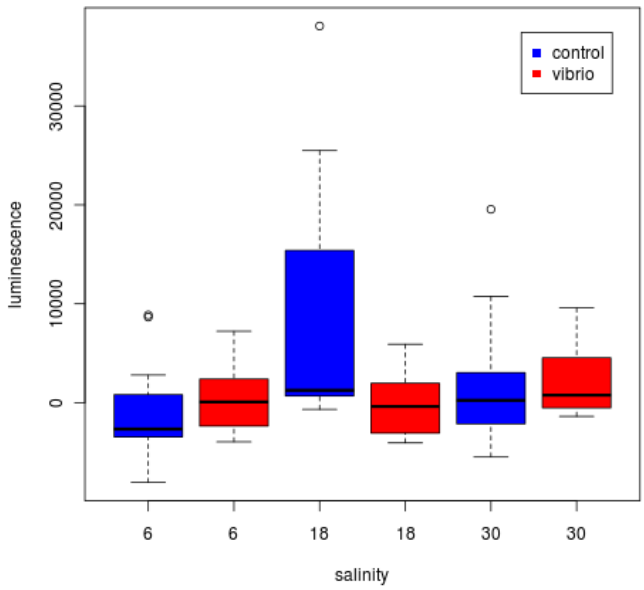
Figure 3.4: These boxplots show the proportion of lymphocytes, i.e. cells from the adaptive immune system, in the blood that are in the mitosis and cell division (G2M) stage, one and eight days after infection. The blue bars again represent the fish from the control treatments, whereas the red bars stand for the fish that were infected with *Vibrio*.

Respiratory burst

Table 3.3 shows the results of the ANOVAs and post-hoc tests for the respiratory burst of the monocytes in the blood of the fish. Significant effects of salinity, timepoint and salinity*timepoint interaction were found. In the according Figure 3.5 one can see that in timepoint 1 the fish in the highest salinity have up-regulated the phagocytosis rate and thus the activity of their monocytes. The post-hoc TukeyHSD test shows that there is only a salinity effect in timepoint 1, where salinity 30 has significantly higher values than both salinity 6 and 18. One has to consider that the range of the y-axis is different between the plots for the two timepoints - in the plot for the second timepoint (Figure 3.5(b)) the y-axis has a much smaller range. Thus the activity of the monocytes of all the salinities in timepoint 2 are around the activity of salinity 6 and 18 in timepoint 1 (Figure 3.5(a)). *Vibrio* has no effect on the respiratory burst.



(a) Phagocytosis rate in the blood of the fish one day after infection.



(b) Phagocytosis rate in the blood of the fish eight days after infection.

Figure 3.5: The phagocytosis rate, i.e. the activity of the monocytes, in the blood of the fish one and eight days after infection is shown. The blue bars again represent the fish from the control treatments, whereas the red bars stand for the fish that were infected with *Vibrio*.

Table 3.3: The results of the statistical tests for the phagocytosis rate of the monocytes in the blood of the fish are shown. Timepoint 1 is defined as one day after infection, whereas timepoint 2 represents the data from eight days after infection. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.5 in the Appendix.

parameter	**p-value**
ANOVAs	
salinity	**0.0030**
vibrio	0.2899
timepoint	**6.52E-05**
salinity*vibrio	0.9989
salinity*timepoint	**1.001E-05**
vibrio*timepoint	0.3856
salinity*vibrio*timepoint	0.7705
TukeyHSD	
18PSU-6PSU	0.7971
30PSU-6PSU	**0.0299**
30PSU-18PSU	**0.0040**
timepoint1 - timepoint2	**7.84E-05**
18:tp1 - 6:tp1	0.9267
30:tp1 - 6:tp1	**4.09E-04**
6:tp2 - 6:tp1	0.9791
18:tp2 - 6:tp1	0.9959
30:tp2 - 6:tp1	0.1086
30:tp1 - 18:tp1	**2.71E-05**
6:tp2 - 18:tp1	1.0
18:tp2 - 18:tp1	0.9986
30:tp2 - 18:tp1	0.5908
6:tp2 - 30:tp1	**0.0014**
18:tp2 - 30:tp1	**3.43E-04**
30:tp2 - 30:tp1	**1E-07**
18:tp2 -6:tp2	0.9998
30:tp2 -6:tp2	0.7028
30:tp2 - 18:tp2	0.3825

Gene expression assay

The housekeeping gene, ubiquitin, was generally expressed to a higher extent than the tested immune genes, i.e. the C_T value was lower. Hence, a more negative -ΔC_T for *Vibrio* infected fish, compared to the control fish, always indicates a down-regulation, whereas a higher -ΔC_T indicates that the gene expression was up-regulated.

Due to high CV values (>0.04) three genes had to be completely eliminated from the analyses: hsp, kin and nramp. Additional measurements could have enabled to identify outliers in order to exclude them, but because of the lack of time this was not done. All gene expression measurements of one control sample from 6 PSU had to be excluded, because the reverse transcription had not worked properly. Thus all genes had a maximum of 4 replicates for the control fish in the low salinity. In the first QPCR run (where genes C3, grcsf, IL, TLR and tnf were measured) a *Vibrio* sample from 6 PSU had to be additionally excluded, due to high amplification in the RNA control. In the second run (where genes cf, hsp, kin, LA and nramp were measured) one sample for both control and *Vibrio* treatment from 18 PSU and two samples for each control and *Vibrio* treatment from 30 PSU had to be excluded, because the gDNA digestion had not worked properly. Further exclusions of single gene measurements had to be done due to high CV values. This resulted in the replicate numbers shown in Table 3.4. All measurements had at least three replicates and thus were used for the analyses.

Table 3.4: The number of replicates of the treatments that were achieved for the single genes in the QPCR assay of Experiment 1.

gene	**6 PSU**		**18 PSU**		**30 PSU**	
	control	*Vibrio*	control	*Vibrio*	control	*Vibrio*
C3	4	4	5	4	4	5
grcsf	3	4	4	3	5	5
IL	4	4	4	5	5	5
TLR	4	4	4	5	5	5
tnf	4	4	4	5	5	5
cf	4	5	4	4	3	3
LA	4	3	4	4	3	3

The MANOVA results show that salinity and *Vibrio* significantly affect the gene expression, as does the interaction between salinity and *Vibrio* (Table 3.5). The genes that are influenced by salinity are grcsf, IL and tnf. cf, grcsf, IL and LA are up-regulated compared to the control if the fish are infected with the bacteria. The gene responsible for the significant salinity* *Vibrio* interaction is grcsf. Before conducting post-hoc tests, a similarity percentage test (SIMPER) was done to look at the contribution of the single genes to the found effects, i.e. to find the genes that were mainly responsible for the difference between the treatments. All genes more or less contributed to the effect to the same amount (data not shown) and thus all genes were tested with TukeyHSD. Because not a single gene was responsible for a major percentage of the effect, but all genes contributed to a certain extent, some significances were lost when the genes were tested separately. To compensate for this, the significance level α was raised from 0.05 to 0.1, i.e. all values below 0.1 were considered significant. The results for the post-hoc tests can be seen in Table 3.5. In all three genes that contributed to the salinity difference (grcsf, IL and tnf) salinities 6 and 30 were different. The p-value for tnf was over 0.1, but it was the lowest of all three salinity combinations which made it clear that, even though it was not significant, this difference was the one that contributed most to the salinity

effect. In the Figures 3.7, 3.8 and 3.10 one can see that the fish from the high salinity (30 PSU) on average have the lowest expression of the three genes. In one case, grcsf, it is even significantly different from the expression of the fish in salinity 18. So there seems to be a negative connection between the expression of these three genes and the salinity levels. The *Vibrio* effect is seen in four genes: cf, grcsf, IL and LA. cf, grcsf and IL have significant p-values in the post-hoc tests and thus are the genes that show the effect clearly, as is seen in the according Figures 3.7, 3.8 and 3.6. All fish that encountered a bacterial infection in the experiment up-regulated these three genes. LA is not significant when isolated from the other genes, but also shows a slight trend in having a higher expression in infected fish (Figure 3.9). The significant interaction between salinity and *Vibrio* is solely due to the gene grcsf. Here one can see that, while the gene expressions are not significantly different between the fish from the different salinities in the control treatment, the up-regulation of the gene upon *Vibrio* infection decreases with increased salinity, i.e. fish from 6 and 18 PSU have a higher grcsf expression than fish from salinity 30.
The relative quantity (RQ) values for the tested genes in the different salinities are shown in Table 3.7. Only values over 2, i.e. a doubling of the expression in the *Vibrio* treatment, and below 0.5, i.e. a halving of the expression, are considered to be a substantial change in gene expression. The two very high values are due to a very drastic up-regulation of the gene in one individual. Without this individual there is still a high up-regulation, but not to the extent as shown in the Table. In the low salinity, the infected fish down-regulate the gene C3, whereas in the other salinities the gene expression is up-regulated when the fish are challenged with *Vibrio*. The genes grcsf and IL are considerably up-regulated in the 6 and 18 PSU treatments, IL is additionally up-regulated in fish from 30 PSU. This enforces the results of the post-hoc tests for grcsf in Table 3.6, where we saw that fish from the two lower salinities up-regulate the gene expression to a higher extent than fish in a high salinity when they encouter a bacterial infection. The gene TLR is also substantially influenced by the bacterial infection, but only in the intermediate and high salinities.
The boxplots of the non-significant C3 and TLR can be found in the Appendix.

To make a connection between the gene expression and the immune parameter measurements, correlation analyses were done for all individuals that were used in both assays. The correlation results are listed in Table 3.8. The gene C3, although not significant for salinity or *Vibrio*, seems to have an effect on the amount of immune cells (innate and adaptive) in the blood. If we look at the correlation coefficient (cor) we can see that C3 positively correlates with the amount of lymphocytes. Furthermore, grcsf and IL negatively correlate with the amount of lymphocytes in the head kidney. Additionally, they have a positive correlation with the activity of the lymphocytes. The last found correlation is a positive correlation between the gene expression of cf and the activity of the lymphocytes in the head kidney.

Table 3.5: The results of the MANOVA for the gene expression assay of Experiment 1 are shown. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.6 in the Appendix.

gene	**salinity**	**vibrio**	**salinity*vibrio**
over all	**0.0034**	**0.0019**	**0.0084**
C3	0.2278	0.9128	0.9372
cf	0.6170	**0.0342**	0.8809
grcsf	**3.39E-04**	**5.4E-06**	**0.0032**
IL	**0.0377**	**0.04512**	0.9132
LA	0.3859	**0.0145**	0.1399
TLR	0.7799	0.2358	0.8786
tnf	**0.0158**	0.3836	0.3887

Table 3.6: The results of the post-hoc TukeyHSD tests of the gene expression of Experiment 1. For these tests the significance level α was raised to 0.1. Significant p-values are highlighted with **bold** letters.

		cf	**grcsf**	**IL**	**tnf**	**LA**
salinity	18-6		0.1451	**0.0937**	0.3398	
	30-6		**1.36E-04**	**3.33E-05**	0.1823	
	30-18		**0.0144**	**0.0050**	0.9259	
vibrio	vibrio-control	**0.0101**	**1.83E-05**	**0.0019**		0.3103
salinity *vibrio	18:control - 6:control		0.9492			
	30:control - 6:control		0.1925			
	6:vibrio - 6:control		**0.0143**			
	18:vibrio - 6:control		0.1066			
	30:vibrio - 6:control		1.0			
	30:control - 18:control		0.5643			
	6:vibrio - 18:control		**0.0010**			
	18:vibrio - 18:control		**0.0128**			
	30:vibrio - 18:control		0.9662			
	6:vibrio - 30:control		**2.09E-05**			
	18:vibrio - 30:control		**3.49E-04**			
	30:vibrio - 30:control		0.1452			
	18:vibrio - 6:vibrio		0.9607			
	30:vibrio - 6:vibrio		**0.0031**			
	30:vibrio - 18:vibrio		**0.0395**			

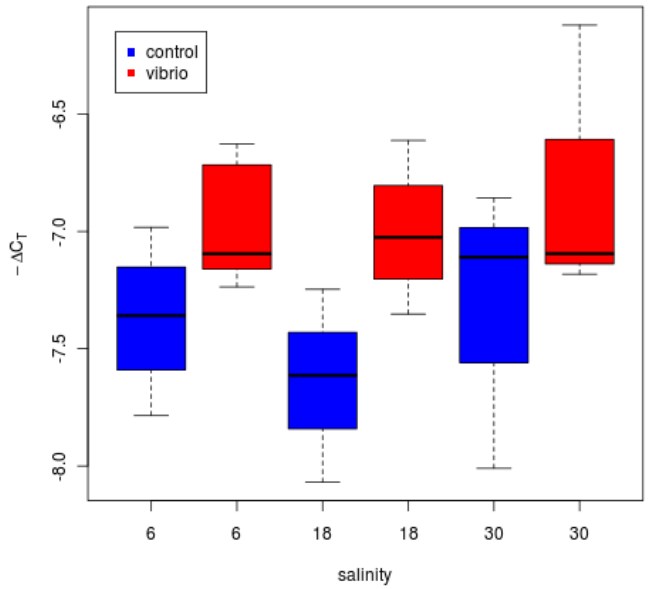


Figure 3.6: The relative activity ($-\Delta C_T$) of the immune gene cf one day after infection for Experiment 1 is shown.

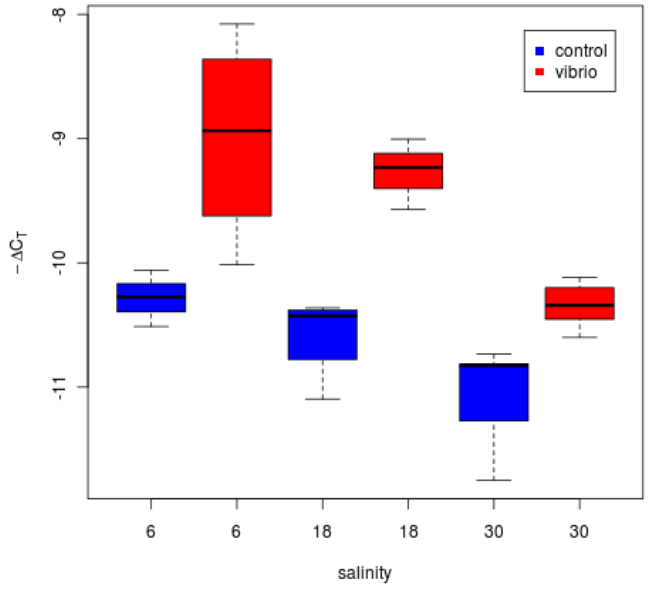


Figure 3.7: The relative activity ($-\Delta C_T$) of the immune gene grcsf one day after infection for Experiment 1 is shown.

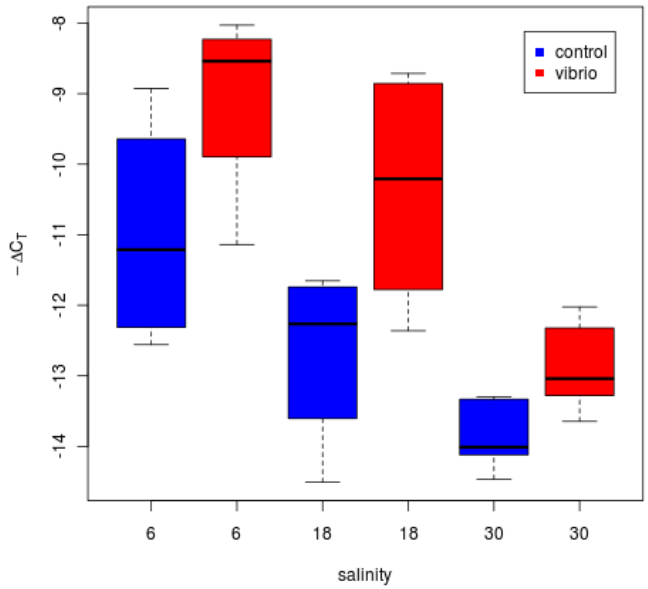


Figure 3.8: The relative activity ($-\Delta C_T$) of the immune gene IL one day after infection for Experiment 1 is shown.

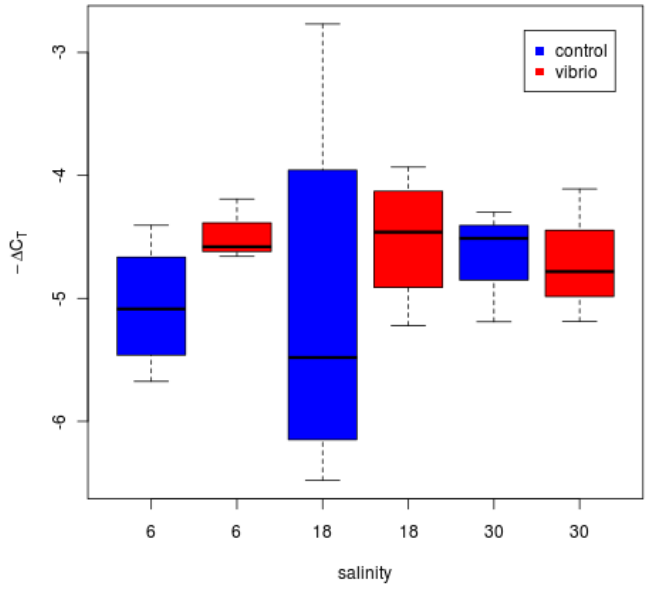


Figure 3.9: The relative activity ($-\Delta C_T$) of the immune gene LA one day after infection for Experiment 1 is shown.

Table 3.7: Relative quantity (RQ) values of the tested genes for the mature fish in three different salinities. Values over 2, i.e. over double amount of expression in the *Vibrio* treatment compared to the control fish, are highlighted in **bold** letters.

salinity	**6**	**18**	**30**
C3	**0.4013**	**1000.1292**	**269.3135**
cf	1.3435	1.5846	1.5282
grcsf	**2.7514**	**2.5100**	1.6779
IL	**4.8701**	**7.6514**	**2.1579**
LA	1.5171	1.5227	1.0310
TLR	1.5120	**7.5570**	**2.1342**
tnf	0.7606	1.7816	1.0463

Table 3.8: The results of the Pearson correlation of the single genes with the different immune parameters are shown for Experiment 1. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.7 in the Appendix.

	head kidney			***blood***			
p-value *cor*	**lympho-cytes** (N=29)	**mono-cytes** (N=29)	**G2M** (N=29)	**lympho-cytes** (N=29)	**mono-cytes** (N=29)	**G2M** (N=29)	**luminescence** (N=29)
C3(N=26)	0.3903 0.1758	0.1246 -0.3090	0.7804 0.0575	**0.0126** **0.4824**	**0.0371** **-0.4109**	0.4946 -0.1402	0.692 -0.0816
cf(N=22)	0.5411 -0.1377	0.4602 0.1660	**0.0297** **0.4639**	0.8614 -0.0395	0.5804 0.1247	0.8208 0.0513	0.6993 0.0873
grcsf(N=24)	**0.0023** **-0.5931**	**6.38E-04** **0.6467**	**0.0041** **0.5644**	0.1841 -0.2806	0.0738 0.3716	0.3728 0.1904	0.0803 -0.3641
IL(N=27)	**0.0127** **-0.4732**	**0.0038** **0.5377**	**0.0034** **0.5434**	0.2052 -0.2518	0.0571 0.3705	0.513 0.1316	0.0561 -0.3719
LA(N=12)	0.8018 0.0583	0.7223 -0.0825	0.458 0.1712	0.3283 0.2243	0.4823 -0.1622	0.4884 0.1600	0.8365 -0.0480
TLR(N=27)	0.0758 -0.3474	0.3083 0.2036	0.0779 0.3451	0.6515 0.0911	0.8577 0.0362	0.9835 -0.0042	0.5295 -0.1265
tnf(N=27)	0.3168 -0.2001	0.5521 0.1197	0.7289 0.0699	0.8893 -0.0281	0.4816 0.1414	0.6633 -0.0878	0.629 -0.0974

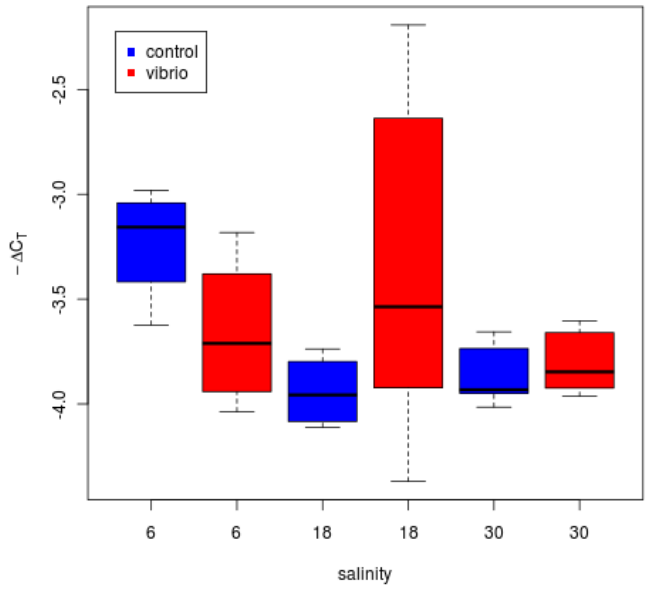


Figure 3.10: The relative activity ($-\Delta C_T$) of the immune gene tnf one day after infection for Experiment 1 is shown.

3.2 Experiment 2 - Juveniles

Gene expression assay

C3 could not be normalised through BoxCox transformation, due to the negative values. Therefore, the SIMPER data set for C3 was taken, where all negative values were eliminated by addition of a constant, but the missing values were left in. Normalisation could then be achieved through a log transformation. Because no data was available for 30 PSU in timepoint 2, two different MANOVAs were conducted. First, an analysis was done, where the data from timepoint 2 was excluded, to check for a difference between the three salinities (Table 3.9). Then the data for 30 PSU was excluded from the data set for the second analysis that included timepoint 2 (Table 3.10). The subsequent SIMPER analyses (of the fourth root transformed data) revealed that all genes contributed to an approximately equal extent to the differences found between the treatments (data not shown). So again, for all genes individual post-hoc tests were performed.
No gene had to be completely removed from the analysis due to low CV values, as was the case for the adult fish, but some genes had only four replicates for certain treatments. This was the case in C3, cf, hsp, kin and LA for salinity 6 and *Vibrio* infection at timepoint 1, where in each case one replicate had to be excluded, so that four replicates were left. For hsp, an additional replicate for the control treatment in salinity 30 at timepoint 1 had to be excluded, thus, again, four replicates remained.
The MANOVA that included all three salinities showed that for timepoint 1 only salinity had an effect. No *Vibrio* effect and no significant interaction was found. The salinity difference was due to the genes C3 and tnf, where the post-hoc tests (Table 3.11) show that

salinity 30 was significantly different from salinity 6 and also tended to be different from the intermediate salinity 18. The Figures 3.11 and 3.17 reveal that the gene expression of C3 and tnf are up-regulated with higher salinities.
The results of the second MANOVA, where 30 PSU was excluded and timepoint 2 was included, show that salinity and timepoint of dissection have a significant effect on the activitiy of the immune genes. grcsf is the gene that accounted for the salinity difference, whereas C3, grcsf, hsp, IL and TLR were responsible for the effect of timepoint. Figure 3.12 shows that the gene expression of grcsf in 6 PSU is up-regulated compared to 18 PSU. Furthermore, in Figures 3.11, 3.12, 3.13, 3.14 and 3.16 one can see that in all significant genes a down -regulation took place between one and five days after infection. The results of the post-hoc tests are shown in Table 3.12, but do not reveal additional information, since the significant effects both only had two levels in this MANOVA. In both tests, no significant effect of *Vibrio* infection was found.
All boxplots of the non-significant genes can be found in the Appendix.

The RQ values for the genes in the juvenile fish are shown in Table 3.13. Again, only values over 2 and below 0.5 are considered to be substantial changes. C3 is significantly up-regulated in 18 PSU one day after the fish encountered the infectious bacteria. IL is also substantially up-regulated in the medium salinity treatment, one day after infection. Furthermore, TLR is up-regulated in the 18 PSU and 6 PSU treatment one day after infection and is down-regulated in 18 PSU five days post-infection.

Table 3.9: The results of the MANOVA for the gene expression of fish from 6, 18 and 30 PSU for the data from timepoint 1 of Experiment 2 are shown. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.8 in the Appendix.

gene	salinity	vibrio	salinity*vibrio
over all	**0.0029**	0.4180	0.1828
C3	**0.0186**	0.1821	0.8805
cf	0.1197	0.4981	0.5019
grcsf	0.1599	0.9224	0.7786
hsp	0.6074	0.6610	**0.0120**
IL	0.8857	0.6967	0.3952
kin	0.9996	0.7423	0.4083
LA	0.8663	0.3934	0.4884
nramp	0.6314	0.8852	**0.0169**
TLR	0.4766	0.3671	0.3605
tnf	**0.0408**	0.3531	0.9753

Table 3.10: The results of the MANOVA for the gene expression of fish from 6 and 18 PSU including data from both timepoints of dissection of Experiment 2 are shown. Significant p-values are highlighted with **bold** letters. For more detailed results see Table 7.9 in the Appendix.

gene	salinity	vibrio	timepoint	salinity*vibrio	salinity*timepoint	vibrio*timepoint	salinity*vibrio*timepoint
over all	**0.0061**	0.9265	**8.61E-06**	0.8080	0.0782	0.0972	0.5101
C3	0.4070	0.7611	**5.3E-04**	0.9783	0.9056	0.1475	0.8192
cf	0.6664	0.2617	0.7479	0.4713	**0.0349**	0.2620	0.8507
grcsf	**0.0317**	0.7236	**0.0010**	0.7551	0.7797	0.9482	0.6075
hsp	0.9402	0.8819	**0.0098**	0.3820	0.5413	0.0772	0.1538
IL	0.7952	0.4003	**9.84E-04**	0.9961	0.5112	0.9880	0.2944
kin	0.1522	0.8066	0.7930	0.3558	0.1562	0.4592	0.4892
LA	0.1409	0.7817	0.7372	0.7320	**0.0407**	0.6818	0.2912
nramp	0.8106	0.8184	0.2944	0.9355	0.5149	0.0776	0.4525
TLR	0.4849	0.9969	**2.43E-08**	0.5666	0.1639	**0.0115**	0.6848
tnf	0.1866	0.3426	0.1373	0.3185	0.7992	0.9884	0.2777

Table 3.11: The results of the post-hoc TukeyHSD tests of the gene expression of fish from 6, 18 and 30 PSU for the data from timepoint 1 of Experiment 2. For these tests the significance level $alpha$ was raised to 0.1. Timepoint 1 (tp1) refers to one day after infection and timepoint 2 (tp2) represents the data from five days after infection. Significant p-values are highlighted with **bold** letters.

		C3	tnf
salinity	18-6	0.7496	0.4954
	30-6	**0.0710**	**0.0098**
	30-18	**0.0152**	0.1233

Table 3.12: The results of the post-hoc TukeyHSD tests of the gene expression of fish from 6 and 18 PSU including data from both timepoints of dissection of Experiment 2. For these tests the significance level $alpha$ was raised to 0.1. Timepoint 1 (tp1) refers to one day after infection and timepoint 2 (tp2) represents the data from five days after infection. Significant p-values are highlighted with **bold** letters.

		C3	grcsf	hsp	IL	TLR
salinity	18-6		**0.0210**			
timepoint	2-1	**5.34E-04**	**7.81E-04**	**0.0099**	**8.10E-04**	**0**

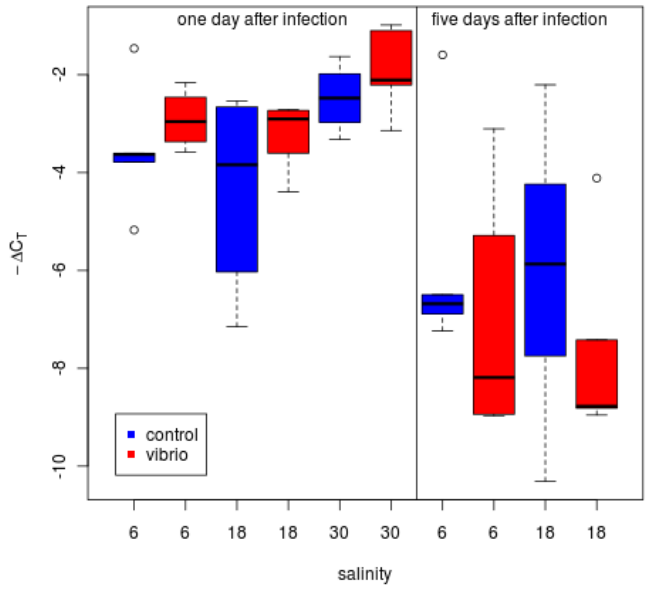


Figure 3.11: The relative activity ($-\Delta C_T$) of the immune gene C3 one and five days after infection for Experiment 2 is shown.

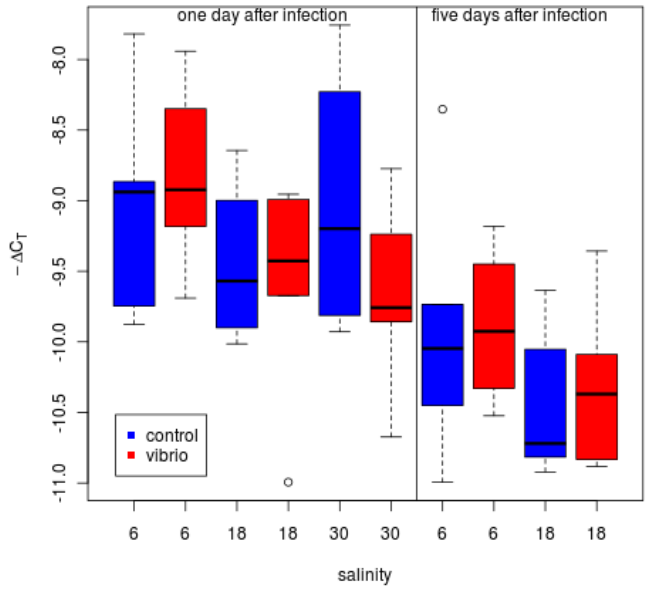


Figure 3.12: The relative activity $(-\Delta C_T)$ of the immune gene grcsf one and five days after infection for Experiment 2 is shown.

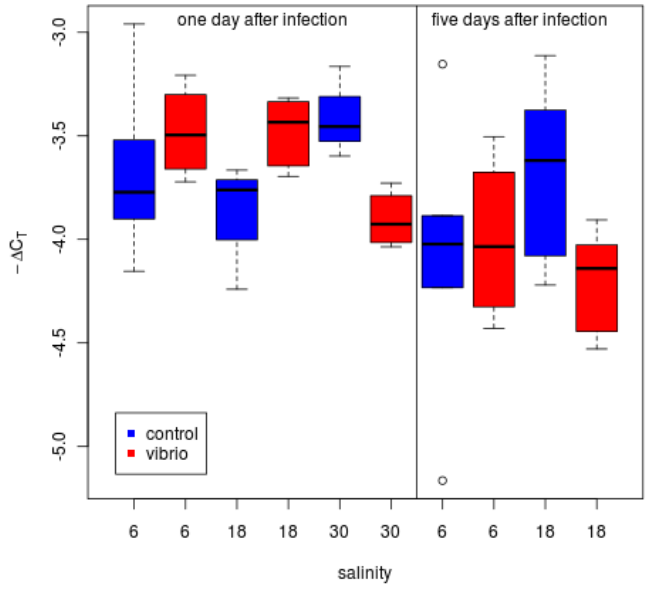


Figure 3.13: The relative activity $(-\Delta C_T)$ of the immune gene hsp one and five days after infection for Experiment 2 is shown.

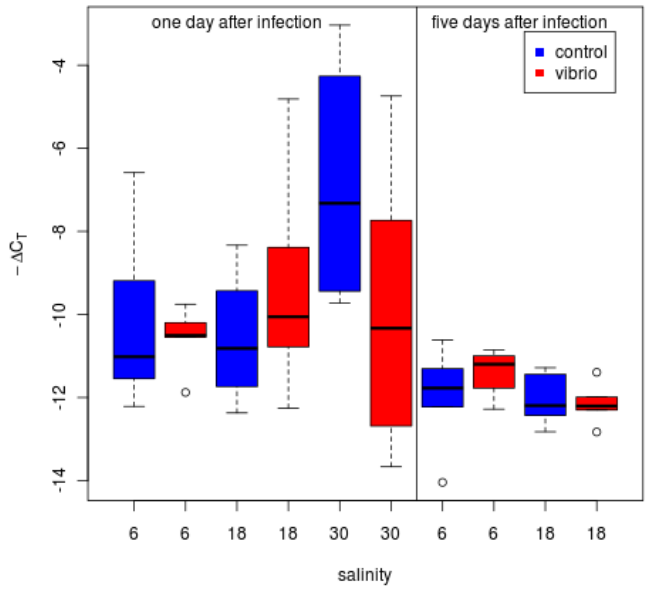


Figure 3.14: The relative activity ($-\Delta C_T$) of the immune gene IL one and five days after infection for Experiment 2 is shown.

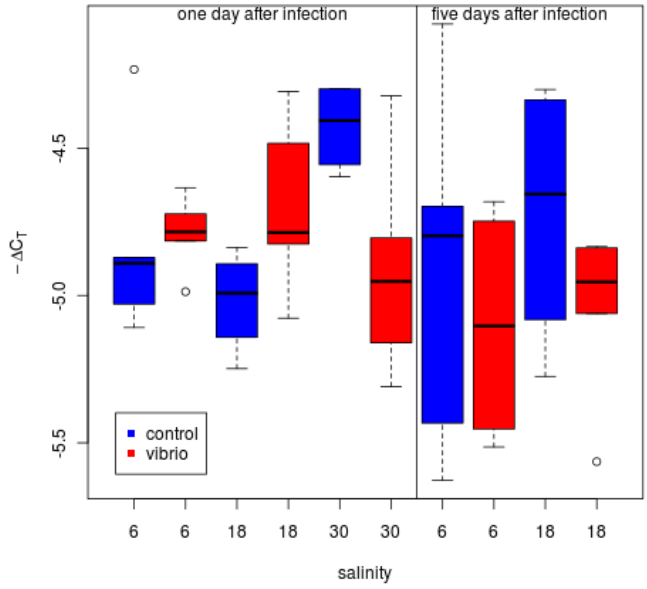


Figure 3.15: The relative activity ($-\Delta C_T$) of the immune gene nramp one and five days after infection for Experiment 2 is shown.

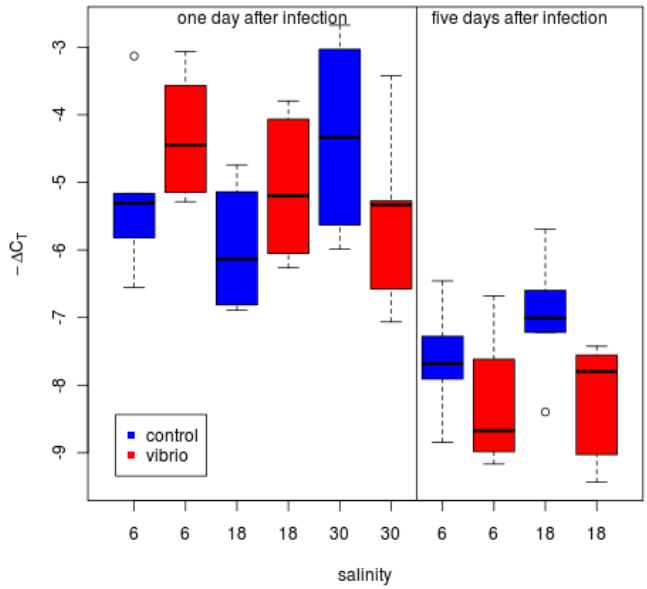


Figure 3.16: The relative activity ($-\Delta C_T$) of the immune gene TLR one and five days after infection for Experiment 2 is shown.

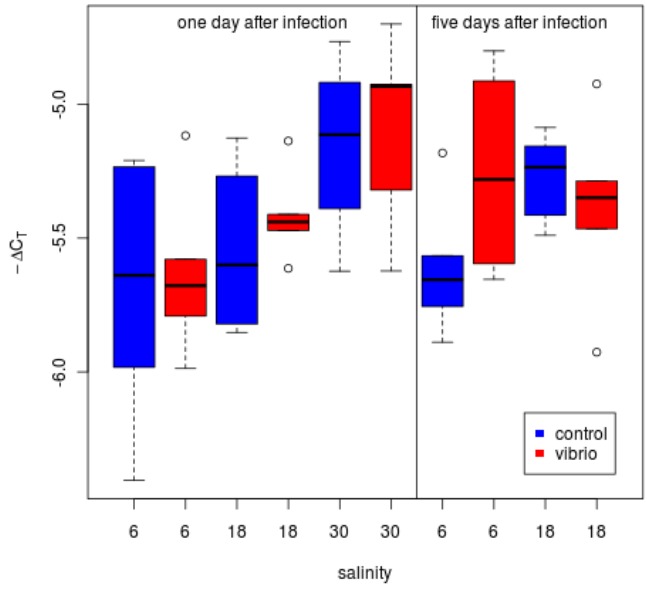


Figure 3.17: The relative activity ($-\Delta C_T$) of the immune gene tnf one and five days after infection for Experiment 2 is shown.

Table 3.13: Relative quantity (RQ) values of the tested genes for the juvenile fish in three different salinities. Values over 2, i.e. over double amount of expression in the *Vibrio* treatment compared to the control fish, are highlighted in **bold** letters.

salinity	**6**	**6**	**18**	**18**	**30**
days after infection	one	five	one	five	one
C3	1.6376	1.7308	**2.2991**	0.9462	1.7077
cf	1.2999	1.7072	0.9371	1.4075	1.1934
grcsf	1.2850	1.0817	0.9997	1.1797	0.7049
hsp	1.1443	1.0982	1.3015	0.7033	0.7131
IL	0.8027	1.6215	**12.3608**	0.9775	0.9952
kin	1.1706	1.2429	0.8308	1.1268	0.9321
LA	0.8601	1.1313	1.0997	0.9695	0.7906
nramp	1.0299	0.9141	1.2717	0.8140	0.7369
TLR	**2.2235**	0.8157	**2.3540**	**0.4806**	0.6522
tnf	1.0676	1.3184	1.1011	0.94723	1.0642

4

Discussion

Our experiments that simulated a realistic scenario of salinity change in the Baltic Sea coupled with infections of *Vibrio spp.* show that pipefish activate their immune system as a response to salinity changes. Thus they are clearly stressed and energy that is used for this reaction upon environmental change is not available for the immune response upon subsequent bacterial infections. This leads to a delayed immune activation that gives the bacteria enough time to harm the fish and possibly inflict damage that is lethal.
The effects of the different parameters are summarised in Tables 4.1, 4.2 and 4.3.

4.1 Salinity effect

Pipefish are particularly stressed in the low salinity (6 PSU), what is seen in the up-regulation of monocyte production in the head kidney of the pipefish compared to ambient natural salinity one day after infection. In the blood, both fish from salinity 6 and 30 have a higher amount of monocytes. The preceding *Vibrio* exposure is ruled out to have induced this immediately visible change, as one day is not enough to synthesise the needed cells and transport them into the blood system. Hence, the cell count in the blood shows that fish in both changed salinities (high and low) are stressed, but that the fish exposed to the high salinity environment (30 PSU) react only on a short time scale to the increase of salinity (the reaction is seen in the blood, but no new cells are being produced in the head kidney). The monocyte count is lower in all salinities at the second timepoint, what implies that the adaptive immune system has been activated.
At timepoint 2 (Figure 3.2(b)) fish from 18 PSU have the highest lymphocyte count due to an activation of the adaptive immune defence. The fish exposed to high or low salinity, on the other hand, have a higher monocyte count. This pattern suggests that the adaptive immune reaction is delayed or, alternatively, the innate immune reaction is prolonged in pipefish under salinity stress. Hence, environmental stress can have a retarding effect on the specific immune defence and thus on the establishment of immune memory, what makes the fish more probable to suffer severe consequences of an infection that normally can efficiently be combated.

Table 4.1: Direction of the effects found in the immune parameters for Experiment 1. The direction of the effect is always relating to the first of the two treatments that are being compared. Brackets "[ ]" are used to show the timepoint in which the effect was significant, if it only occurred in one timepoint.

parameter	**organ**	**salinity**			***Vibrio***	**timepoint**
		6-18	30-18	6-30	*Vibrio*-control	tp2-tp1
monocyte count	head kidney	⇑		⇑	⇑[tp1],⇓[tp2]	⇓
	blood	⇑	⇑			⇑
lymphocyte activity	head kidney	⇓[tp1]	⇓[tp1]		⇑	⇓
	blood	⇑			⇑	
monocyte activity	blood		⇑	⇓		⇓

Table 4.2: Direction of the effects found in the gene expression for Experiment 1. The direction of the effect is always relating to the first of the two treatments that are being compared. Parentheses "( )" show that the effect was not significant in the post-hoc test.

gene	**salinity**			***Vibrio***
	6-18	30-18	6-30	*Vibrio*-control
cf				⇑
grcsf		⇓	⇑	⇑
IL	⇑	⇓	⇑	⇑
LA				(⇑)
tnf			(⇑)	

In the head kidney, the place where the cells undergo clonal propagation, the lymphocyte activity in fish from the ambient salinity 18 is overall highest one day after infection. This shows that the activity of the adaptive immune cells is first down-regulated under salinity stress, i.e. in fish from salinities 6 and 30, because the energy is needed for the production of innate immune cells (monocytes). Eight days after infection, all fish have down-regulated the lymphocyte activity, but the fish that experienced salinity stress have a slightly, but not significantly, higher rate of lymphocytes that are in the proliferation phase than the fish from 18 PSU. This suggests that the up-regulation of lymphocyte activity in the environmentally stressed fish is retarded. In the blood, the lymphocytes of the fish from the low salinity are the most active at timepoint 1 and salinity 30 seems to have intermediate activitiy (TukeyHSD p-values in Table 3.2). Thus, low salinity appears to be more stressful than a high salinity, which in turn (and intuitively) is more stressful than the habitual intermediate salinity 18.

The significantly higher activity of the monocytes in the blood of the fish kept at 30 PSU matches well with the results of the proportion of monocytes in the blood and head kidney. The fish from the high salinity up-regulate their immune system already before

Table 4.3: Direction of the effects found in the gene expression for Experiment 2. The direction of the effect is always relating to the first of the two treatments that are being compared.

all salinities, only timepoint 1

gene	**salinity**			***Vibrio***
	6-18	30-18	6-30	*Vibrio*-control
C3		⇑	⇓	
hsp				⇑(6& 18PSU),⇓(30PSU)
nramp				⇑(6& 18PSU),⇓(30PSU)
tnf			⇓	

only 6 & 18 PSU, both timepoints

gene	**salinity**	***Vibrio***	**timepoint**
	6-18	*Vibrio*-control	tp2-tp1
C3			⇓
grcsf	⇑		⇓
hsp			⇓
IL			⇓
TLR			⇓

the infection (seen in Figure 3.2(a)), but then stop to synthesise new cells (Figure 3.1(a)), while fish from salinity 6 still do. This is revealed in the higher count of immune cells. Thus, we conclude that environmental stress can result in different immune activation patterns and reactions. Whereas the low salinity triggers a long activation of the innate immune system, a high salinity activates the immune system in the short run and thus produces less cells, but yields many more active cells that can fight the infection in the periphery. The latter pattern seems to be more economic regarding the energy needed, since the fish from 30 PSU have the potential to react to a subsequent infection (Figure 3.1(b)). Here, fish from the habitual salinity 18 have a lower monocyte activity at the first timepoint, but overall a higher activity at timepoint 2 (consider scale of luminescence axis). This again shows that fish from 6 and 30 PSU have reacted to the salinity stress, since the reaction is already visible in the blood. Furthermore, it implies that salinity stress leads to a subsequent down-regulation in immune cell acitvity, probably due to the lack of energy for the maintenance of a certain level of activity.

grcsf is down-regulated in fish that were kept at 30 PSU. This explains the stop of production of monocytes that is seen in the monocyte count. As was found in the respiration burst assay, the fish from 30 PSU probably have up-regulated the grcsf gene for a short period that resulted in the highly active monocytes. This up- and down-regulation of the gene grcsf enables a short and efficient activation of the innate immune system. Furthermore, the expression of IL is down-regulated with higher salinity, what shows that the risk for inflammation is lower in high salinity environments. Alternatively, this gene could have been up-regulated immediately after the encounter of changed environmental salinity and then down-regulated again, as was the case for grcsf. Also in the high salinity, the expression of tnf, the gene that is responsible for negative regulation of the immune

reaction, is down-regulated compared to 6 PSU. It has about the level of expression that control fish from salinity 18 have. This implies that the immune system is not very active and thus does not have to be limited.

In the juvenile fish C3 is up-regulated in 30 PSU. It activates the complement system and opsonises bacteria in the blood system of the fish. This enables a fast and short activation of the immune system, as was seen for the mature fish in the high salinity. tnf also has a higher expression in fish from the high salinity, compared to fish from the low salinity. Thus, in contrast to the findings in the adult fish, the immune response of the juveniles is negatively regulated. The immune system is apparently very active to fight the environmental stress and thus a hyperresponsiveness has to be prevented. grcsf is up-regulated in 6 PSU, when both timepoints are included in the analysis. This results in an activation of the innate immune system and thus implies that low salinities are more stressful for the juvenile fish. Whereas the expression of cf and LA are lower in the ambient salinity at timepoint 1, the expression is lower in the low salinity at the second timepoint. This suggests that the fish from 6 PSU have up-regulated their cf on timepoint one as a response to the salinity stress. On timepoint two when a reaction to the *Vibrio* bacteria is visible, the gene can not be up-regulated to the same extent as fish in the ambient salinity do. LA is a gene that belongs to the adaptive immune system and is up-regulated in fish from the low salinity at first, as a reaction to the salinity stress. Hence, at the second timepoint, when fish from 18 PSU up-regulate the expression of LA, the energy to maintain the activity of LA is not available.
All genes that have a significant timepoint effect are down-regulated five days after infection. This overall lower expression shows that the immune system of the juvenile fish reacts quickly and extensively to the salinity stress. This is probably energetically very expensive, so that the immune system can only react on a short time scale.

4.2 *Vibrio* effect

When the fish are challenged with a *Vibrio* infection, the ones in salinity 18 and 30 activate their monocyte proliferation, compared to the control treatment. However, fish from salinity 6 do not react upon *Vibrio* exposure. Organisms suffer a resource allocation trade-off (Sheldon & Verhulst, 1996 and Lochmiller & Deerenberg, 2000), where investment into immune defence is often traded-off with investment into other life-history traits, such as metabolism and reproduction. Hence, pipefish invest major parts of their resources to handle the stress induced by the changes in salinity, at the cost of a decreased amount of resources available for immune defence upon *Vibrio* exposure. Since fish from 30 PSU react on a shorter time scale to the salinity stress (as seen above), they have still enough energy to react upon the *Vibrio* exposure that occurs only shortly after the adaptation to the high salinity. Fish from salinity 6, on the other hand, suffer longer and more intense. One the one hand, this can be due to the salinity stress, such that their maximum immune activation was already reached before they were even infected with the bacteria. On the other hand, this effect could be explained by a higher virulence of the *Vibrio* bacteria in low salinities. Hence, the infection persists longer and the immune

system has to fight longer, so that the reaction can still be seen eight days after infection. Either way, this can have major consequences on fish survival in low salinities upon a virulent *Vibrio* infection.

Pipefish exposed to *Vibrio* had a higher monocyte count at the first, but a higher lymphocyte count at the second timepoint (*Vibrio**timepoint interaction), compared to the control fish. Whereas cells of the innate immune system are of more importance during the early phase of infection, adaptive immune defence is costly and slow in reaction, and only plays a major role in the later phase of the infection, because it needs time to be activated. In addition, up-regulation of one parameter of the immune system can result in down-regulation of another, due to the above mentioned resource allocation trade-off.

The lymphocyte activity in the head kidney was increased shortly after *Vibrio* exposure (timepoint 1), while the fish from the natural salinity, 18, had the highest activity. At timepoint 2 all control fish (regarding *Vibrio* infection) have ceased the lymphocyte activation. However the fish from 6 and 30 PSU have now up-regulated the replication of their lymphocytes. This suggests that fish at the natural salinity have, at the second timepoint, already successfully combated the infection and do not need to send novel replicating lymphocytes to the periphery. Hence, the reaction to the bacteria in 18 PSU has ceased at the timepoint of the second dissection, whereas it is delayed and thus still ongoing in the fish from the other two salinities, because of a lack of energy due to preceding stress or a higher virulence of the infecting agents. The high variation of the activity in infected fish from 6 and 30 PSU suggests that the individual fish can react quite differently, probably based on their previous health condition and experience with bacteria, the amount of nutrition uptake or possibly on the immune characteristics that were inherited from their parents (immune priming, see Grindstaff et al., 2003).

Vibrio infection has an effect on four of the seven tested genes: cf, grcsf, IL and LA. All are up-regulated as a response to the bacterial challenge, what implies that the fish can react promptly to a new threat. The expression of cf is up-regulated when the fish are infected with *Vibrio* to increase blood clotting in order to inhibit blood loss and to decrease the flow speed and thus counteract a distribution of the bacteria in the body, especially to vitally important organs. Furthermore, the expression of grcsf is not up-regulated in the fish from 30 PSU to the same extent as in the other two salinities. This implies that the short, but intensive (very active monocytes), reaction of the fish successfully fights the pathogen, so that the activation of the immune gene is no longer necessary. On the other hand it could suggest that the energy to maintain a certain level of expression is lacking after a very intense activation of the monocytes.
The RQ values show that fish in the medium salinity up-regulate most of the immune genes upon a bacterial infection and thus can react better to the threat than fish from 6 and 30 PSU. C3 is even down-regulated and TLR is not activated in the low salinity. This leads to a delayed immune reaction, as was seen in the cellular immune measurements. The fish from 30 PSU react upon infection, but not to the same extent as fish from 18 PSU, what can also have a retarding effect on the immune reaction.

The juvenile fish show no clear *Vibrio* effect. This may be due to a failed infection

procedure or to the fact that they have never before encountered bacteria of this genus. The young fish cannot react to such a new infection, because they are not fully immunocompetent at the time of birth (Tatner, 1997) and first seem to have to learn how to activate the immune system upon a confrontation with *Vibrio spp.* However, the juvenile fish from the two lower salinities seem to react to a bacterium, in contrast with the fish from the high salinity. This is shown in that the fish from 30 PSU are the only ones that do not up-regulate immune genes, whereas the others do. The two concerned genes are hsp and nramp, which are responsible for chaperone production and macrophage activation. The lack of activation of the immune system in the high salinity may be due to a weakening of the fish in this treatment and thus the selection pressure lead to the high mortality rate that was observed (all juvenile fish in 30 PSU died during the first three days). Another explanation for the high mortality is the high amount of artificial sea salt that was needed to achieve 30 PSU due to the low ambient salinity on Gotland, where Experiment 2 was conducted. The only gene, where a significant interaction between *Vibrio* and timepoint was found, is TLR, which is important for the recognition of pathogens. This gene is up-regulated in infected individuals at the first timepoint and down-regulated five days later. TLR is part of the innate immune system and is activated shortly after an infection in order to recognise the pathogen and mediate the production of the necessary proteins. In a later stage of the infection, genes that belong to the innate immune system are down-regulated to enable an up-regulation of the adaptive immune system.
The RQ values for the juvenile fish show that especially fish from the medium salinity react upon a *Vibrio* infection. TLR is the only gene that is also up-regulated in the low salinity on timepoint one. This suggests that fish from 6 PSU have a slower activation of the immune system upon a bacterial infection, because no other genes than TLR, that are important to fight the bacteria, are yet up-regulated. The fish from 30 PSU do not up-regulate any genes, what could be due to the weak condition of the fish in this treatment.

4.3 Correlation between immune parameters and immune genes

The correlation tests of the gene expression and the immune parameters identified some of the tested genes to be important for the immune reaction at the cellular level. The expression of C3, for example, results in an increase of the release of lymphocytes from the head kidney into the blood. C3 activates the complement system, what suggests that this system plays a role in the transport of the adaptive immune cells to the blood, in order to enable fighting at the periphery. C3 directly opsonises bacteria, i.e. marks the bacterial cells for destruction by phagocytes. Apparently, this gene links the innate to the adaptive immune system. While it performs a step of the innate reaction to a pathogen, it also mediates the activation of the adaptive immune system. grcsf and IL correlate negatively with the amount of adaptive immune cells (lymphocytes) in the head kidney, and at the same time positively correlate with an increased activity of these cells. It is possible that a decrease in amount of lymphocytes acts as a trigger for the expression of grcsf and IL, which then activate the proliferation of more cells. cf positively correlates

with the activity of the lymphocytes in the head kidney. When the lymphocytes are active in the head kidney and preparing to fight the pathogen, cf is expressed and active to prevent meanwhile spreading of the pathogen in the host until the lymphocytes are transported into the blood.

5

Conclusions

Global change affects the oceans in several ways and new environmental conditions are created that pose a stress on the organisms living in the affected areas. Especially subtle dynamics of host-parasite genotype fluctuations can be disrupted by such quick and major changes.
The aim of this study was to look at the immune reaction of the broadnosed pipefish to the *Vibrio* bacterium under different salinity conditions. The results suggest that salinity changes pose a stress to the fish that in response to this activate their immune system. We found two major ways of how salinity stress affects immune defence. A) the immune system is activated for a prolonged time what results in the synthetisation of a high amount of immune cells or B) there is only a short activation yielding very active immune cells. Prolonged activation time of immune cells (A) was shown in lowered salinities, whereas an intense but short activation of immune cells occurred in fish that were exposed to high salinity. Especially a long lasting immune reaction is energetically expensive. The used energy later seems to lack for the reaction to other stressful events, such as bacterial infections, due to a resource allocation trade-off.
In the next decades the Baltic Sea water is predicted to decrease in salinity. This creates the circumstances where pipefish are stressed by the water they swim in and the effect this has on the virulence of *Vibrio spp.* Infections can then not be fought as effectively as under present conditions and thus the death rate of pipefish through bacterial infections, especially vibriosis, and consequences from other pathogens might rise. Of course, long-term consequences of global warming, despite a high speed of change, will allow for adaptations, but the high salinity changes due to strong rainfalls that occur within several hours and especially influence shallow waters at the coasts where pipefish live, makes adapations of the pipefish dubious.
Furthermore, the juvenile fish were found to react quickly to the environmental change, but only on a short time scale. This suggests that the quick reaction is energetically very expensive and thus can not be maintained over a longer period. Hence, a subsequent bacterial infection could be lethal. This could lead to a significantly higher death rate of juvenile fish and thus the whole pipefish population could be endangered.

Salinity change is only part of the future Baltic Sea water under global change, so to understand the full impact of the changing environment on pipefish, more experiments have to be done. Temperature, for example, is predicted to rise and extreme events should become more frequent. Experiments with *S. typhle* and *V. anguillarum* under an extreme temperature event, a heatwave as the one that was experienced in 2003, have already been done by S. Landis (unpublished data). To get an idea how the reactions would be in the wild, where all parameter changes occur simultaneously, an experiment that combines both lower salinity and temperature rise would be the logical follow-up. Furthermore, more immune genes could be added to the gene expression assay, to get a more detailed picture of the reaction on genetic level. Especially genes for the adaptive immune system should be added, since these were largely lacking in the present study.

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6

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7

Appendix

7.1 Establishment of the gene expression assay

Primer design - From the expressed sequence tag (EST) library of *Syngnathus typhle* immune genes (O. Roth & D. Haase, unpublished data) ten genes were randomly chosen. Homologous sequences from other fish species were found with BLAST search and aligned with the pipefish genes in BioEdit. This enabled the more precise location of the encoding region on the target gene. Primers which enclosed these coding regions were then designed using Primer3 v. 0.4.0 (Table 7.1). The amplicons had a length of around 150-200 bp. Additionally, primers were designed for the housekeeping gene ubiquitin which was later used to normalise the gene expression measurements.
We first tried to design the primers on exon-exon boundaries to ensure that no genomic DNA (gDNA) would be amplified during the assay. Unfortunately, this was not possible because the EST library gives no information of where these boundaries are. Furthermore, the immune genes of the pipefish have a rather unique composition and thus these boundaries cannot be identified reliably through alignments with genes of other fish species. Therefore, we decided to perform a gDNA wipeout instead. This step was done just before the reverse transcription of the RNA.

Polymerase chain reaction - A polymerase chain reaction (PCR), with gill samples of pipefish, was conducted for every primer pair to check its functioning. The PCR products were run in a gel and stained with ethidium bromide. Under UV-light the products were made visible and checked for their lengths. The correctly functioning primers, i.e. with only one product of the right length, were used for the quantitative real-time reverse transcription PCR (QPCR) measurements.

Primer efficiency - To obtain optimal gene expression measurements, the primer should work efficiently, whereas an efficiency of 1 means that the amount of DNA is doubled after every cycle. To rule out inefficient primers from further measurements, the efficiencies were calculated from the slopes of the standard curves of each primer, i.e. the slope of the C_T-values (the number of cycles used in the reaction before reaching the linear

amplification phase) from a dilution series. For this purpose, 1 µg RNA from gills was reverse transcribed and 6 dilutions were prepared from the resulting cDNA – 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. These dilutions were then used as templates for the QPCR, each well containing 10µl SYBR Green PCR Master Mix, 10pmol of forward and reverse primer, respectively, and 4µl of template. Each reaction mix was conducted in triplets. Only primers with a slope between 0.8 and 1 and correlations between 0.9 and 1 were used for gene expression measurements.

Table 7.1: Primer sequences for the examined genes. Ubiquitin was used as a reference gene for the calculations of the relative gene expressions.

Gene name	**Symbol**	**Forward Primer Sequence 5'-3'**	**Reverse Primer Sequence 5'-3'**	**Sequence size in bp**
complement component 3	C3	AGACCCCAACATGAAGCAGT	CTCTCTCAATAGGCTCCATGC	190
heat-shock 60kDa protein 1	hsp	GTACGGTCATCATCGAGCAG	AGCACTGTGGCAGTGGTG	177
interleukin 10	IL	TTCCTGACTGCACAGTTGCT	TCTTCGATTGTCTGGTCGAG	187
lymphocyte antigen 75	LA	GCGCGGATATCCTAACCAT	CATGAGTGCCATCGTACCAC	149
toll-like receptor 5	TLR	CACCTGAAGAAACTCCAGCA	GGAGCGCAAATTGTTAGAGC	218
kinesin family member 13b	kin	CGACAACAACCCAGACTCAG	TGTGGGTCTTTGGTGCTG	197
coagulation factor II receptor -like 1	cf	TTACAGAGCGGCCTCACC	TCCAGATGCAAAAGCAGGTC	175
natural resistance-associated macrophage protein	nramp	GAGTGGTTCTGGGCTGTTTC	CAGTAGCACTCGTGCGAAAC	163
granulocyte colony-stimulating factor precursor	grcsf	TTGCAAGGTCTCCAGACAGA	GTTCCTCCTGCTGGCTCTT	178
tumor necrosis alpha-induced protein 8-like 2	tnf	CCTGCTGAAGCTGGTCAAC	TGTTGAGTCCCTTGCAGATG	157
ubiquitin	ubi	CGTGAAGACATTGACGGGTA	GCAGCACCAGATGAAGAGTG	202

Table 7.2: Functions of the used immune genes.

immune gene	function	innate or adaptive?	reference
C3	activation of the complement system, opsonisation of bacteria	innate	NCBI
hsp	chaperone, guides several steps during syhtesis, transportation and degradation of proteins	innate	Chen et al., 1999
IL	anti-inflammatory cytokine	mainly innate	NCBI
LA	endocytic receptor, guides antigens from extracellular space to antigen-processing compartment, reduces proliferation of B lymphocytes	adaptive	UniProtKB
TLR	recognition of pathogen associated molecular patterns (PAMPs) and mediation of production of necessary cytokines	innate	NCBI
kin	intracellular transport of membrane-associated guanylate kinases (MAGUKs)	innate and adaptive	UniProtKB
cf	involved in thrombotic response: blood is clotted to prevent blood loss	innate	wikipedia
nramp	regulates macrophage activation	innate	Goswami et al., 2001
grcsf	support of proliferation and differentiation of hematopoietic cells	innate	Tabbara, 1993
tnf	negative regulator of immune system, prevents hyperresponsiveness	innate and adaptive	UniProtKB
ubi	labels regulatory proteins for degradation	none	Hershko et al., 1998

7.2 Additional plots - Experiment 1

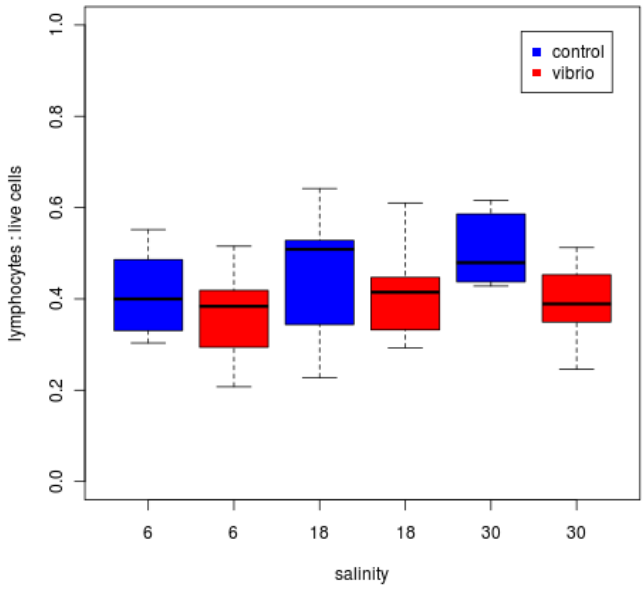
Immune assay

Table 7.3: The degrees of freedom and F values for the results of the statistical tests for the proportion of monocytes in the head kidney and blood of the fish are shown.

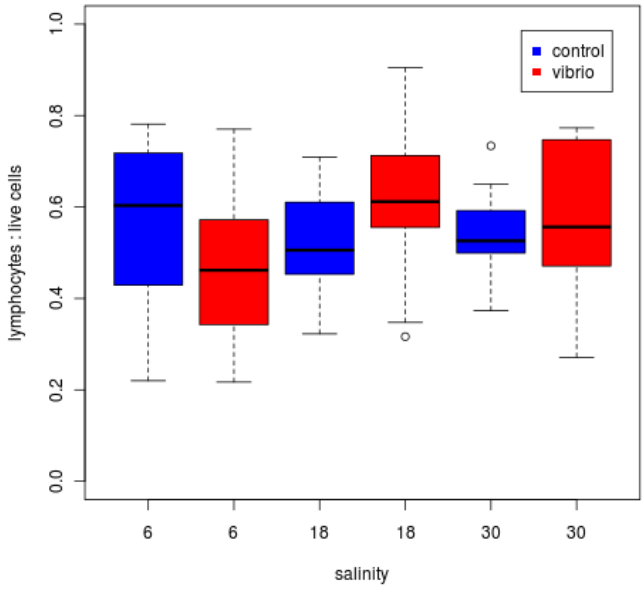
		head kidney	***blood***
parameter	**df**	**F**	**F**
salinity	2	**4.6674**	**9.8454**
vibrio	1	1.0435	0.1321
timepoint	1	**26.2303**	**20.0545**
salinity*vibrio	2	0.0184	0.3903
salinity*timepoint	2	2.7464	0.4634
vibrio*timpeoint	1	**4.2120**	0.2321
salinity*vibrio*timepoint	2	0.7034	0.4888
Residuals head kidney	131		
Residuals blood	130		

Table 7.4: The degrees of freedom and F values for the results of the statistical tests for the proportion of lymphocytes in stage G2M in the head kidney and blood of the fish are shown.

		head kidney	***blood***
parameter	**df**	**F**	**F**
salinity	2	2.8342	**3.7629**
vibrio	1	**18.2983**	**4.5866**
timepoint	1	**109.9190**	0.0185
salinity*vibrio	2	0.3413	0.6619
salinity*timepoint	2	**6.7967**	1.3990
vibrio*timepoint	1	0.6845	2.0179
salinity*vibrio*timepoint	2	0.0714	0.7923
Residuals head kidney	107		
Residuals blood	105		

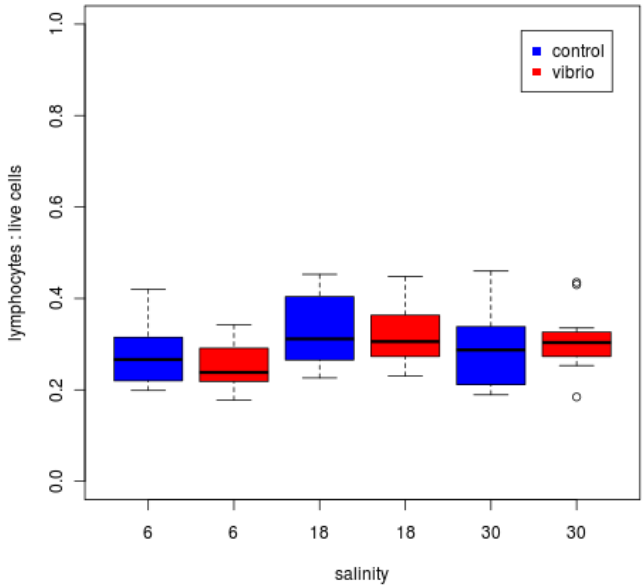


(a) Proportion of lymphocytes in the head kidney of the fish one day after infection.

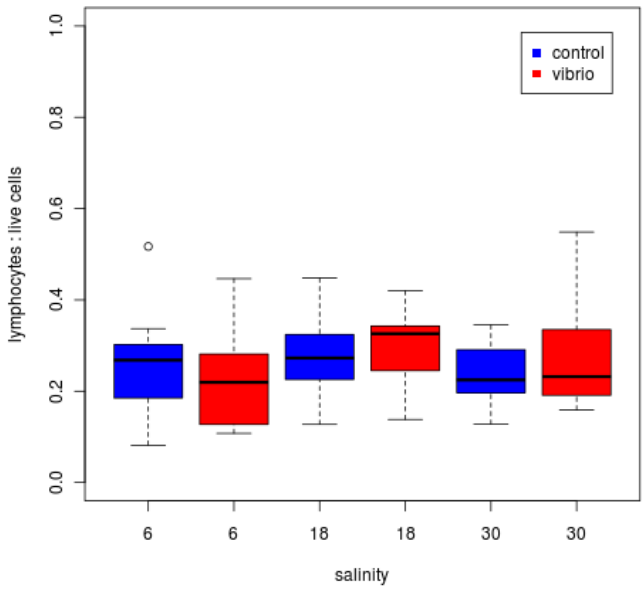


(b) Proportion of lymphocytes in the head kidney of the fish eight days after infection.

Figure 7.1: The proportion of lymphocytes, i.e. the number of lymphocytes divided by the number of total live cells counted, in the head kidney of the fish from the different treatments one and eight days after infection is shown. The blue bars represent fish from the control treatment and the red bars show the data for the fish that were infected with *Vibrio*.

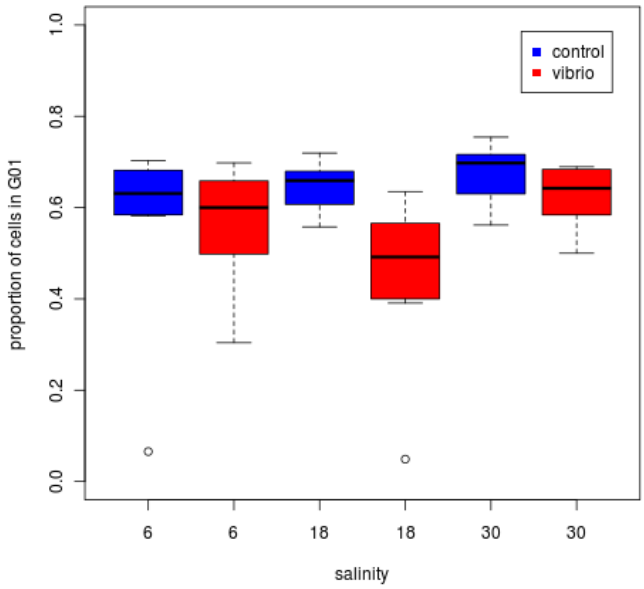


(a) Proportion of lymphocytes in the blood of the fish one day after infection.

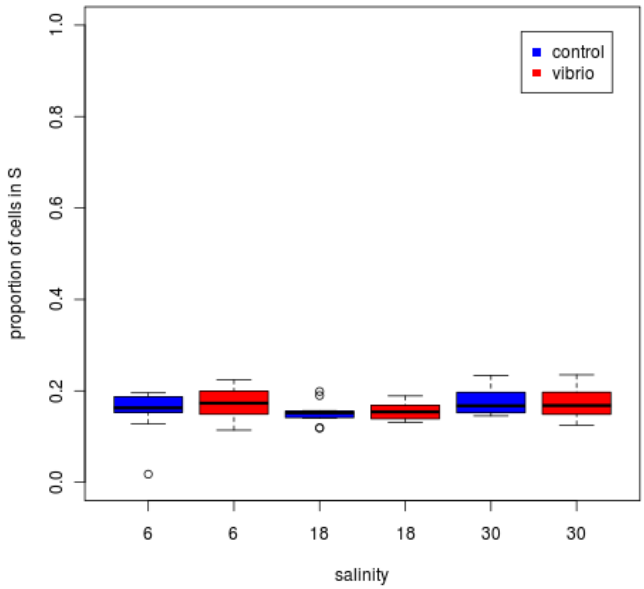


(b) Proportion of lymphocytes in the blood of the fish eight days after infection.

Figure 7.2: The proportion of lymphocytes, i.e. the number of lymphocytes divided by the number of total live cells counted, in the blood of the fish from the different treatments one and eight days after infection is shown. The blue bars represent fish from the control treatment and the red bars show the data for the fish that were infected with *Vibrio*

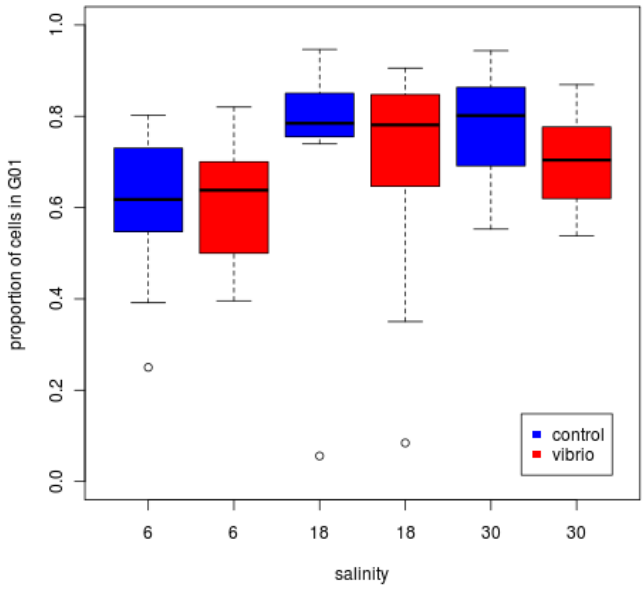


(a) Proportion of lymphocytes in the head kidney that are in the resting stage (G01) one day after infection.

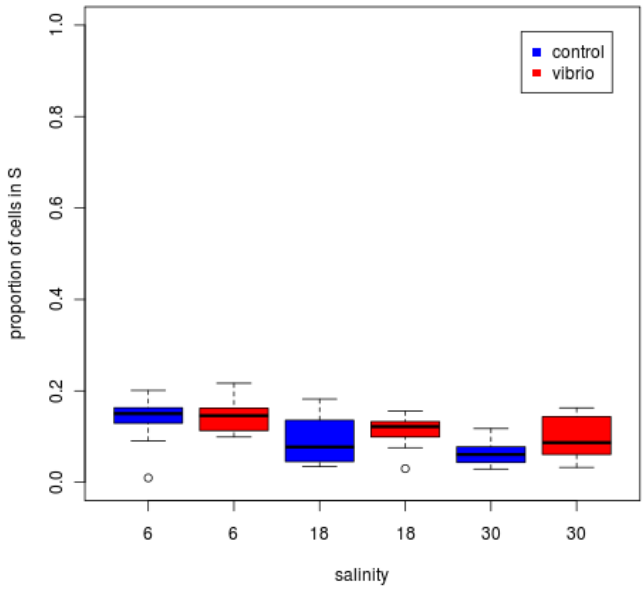


(b) Proportion of lymphocytes in the head kidney that are in the DNA synthesis and chromosome duplication stage (S) one day after infection.

Figure 7.3: The proportion of lymphocytes, i.e. cells from the adaptive immune system, in the head kidney that are in the resting (G01) and DNA synthesis (S) stage are shown. The blue bars again represent the fish from the control treatments, whereas the red bars stand for the fish that were infected with *Vibrio*.



(a) Proportion of lymphocytes in the blood that are in the resting stage (G01) one day after infection.



(b) Proportion of lymphocytes in the blood that are in the DNA synthesis and chromosome duplication stage (S) one day after infection.

Figure 7.4: The proportion of lymphocytes, i.e. cells from the adaptive immune system, in the blood that are in the resting (G01) and DNA synthesis (S) stage is shown. The blue bars again represent the fish from the control treatments, whereas the red bars stand for the fish that were infected with *Vibrio*.

Table 7.5: The degrees of freedom and F values for the results of the statistical tests for the phagocytosis rate of the monocytes in the blood of the fish are shown.

parameter	**df**	**F**
salinity	2	**6.2762**
vibrio	1	1.1355
timepoint	1	**17.8169**
salinity*vibrio	2	0.0011
salinity*timepoint	2	**13.3914**
vibrio*timepoint	1	0.7613
salinity*vibrio*timepoint	2	0.2616
Residuals	78	

Gene expression assay

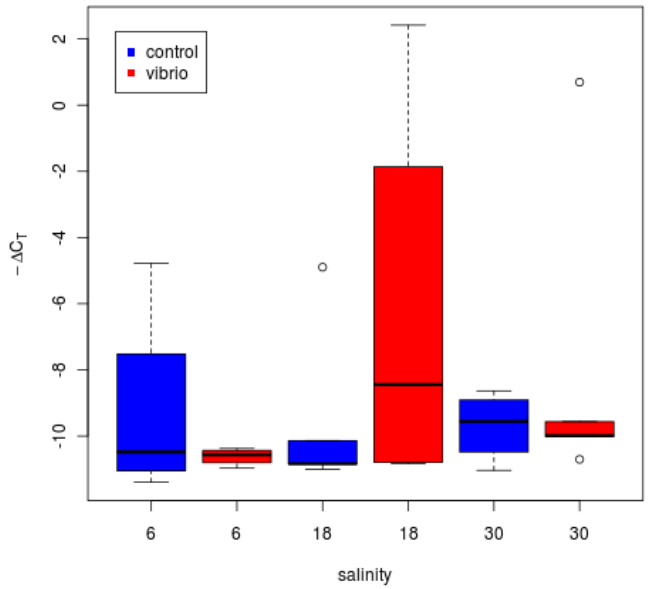


Figure 7.5: The relative activity ($-\Delta C_T$) of the immune gene C3 one day after infection for Experiment 1 is shown.

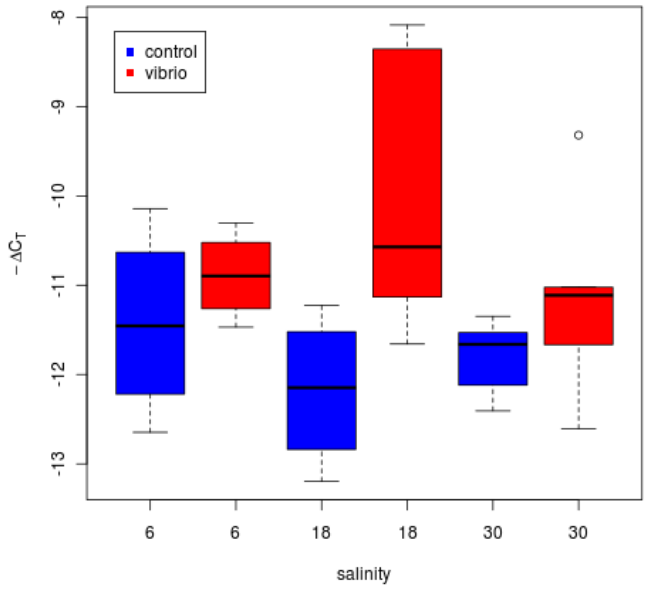


Figure 7.6: The relative activity ($-\Delta C_T$) of the immune gene TLR one day after infection for Experiment 1 is shown.

Table 7.6: The degrees of freedom and F values for the results of the MANOVA for the gene expression assay of Experiment 1 are shown.

gene	**salinity** ***F***	**vibrio** ***F***	**salinity*vibrio** ***F***
over all	**7.731**	**85.044**	**5.868**
C3	1.7514	0.0127	0.0653
cf	0.5098	**6.2197**	0.1287
grcsf	**22.064**	**90.545**	**11.683**
IL	**4.8222**	**5.4053**	0.0918
LA	1.0605	**9.1081**	2.4671
TLR	0.2556	1.6140	0.1313
tnf	**6.8061**	0.8389	1.0515
df	2	1	2
Residuals	9		

7.3 Additional plots - Experiment 2

Gene expression assay

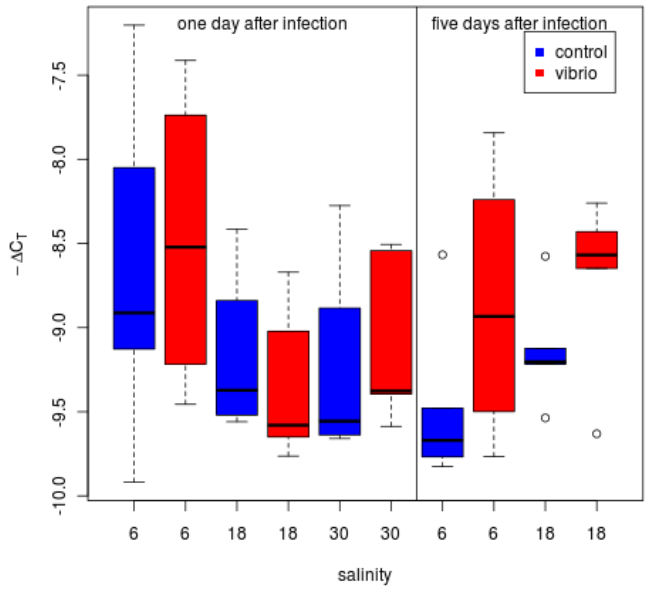


Figure 7.7: The relative activity ($-\Delta C_T$) of the immune gene cf one and five days after infection for Experiment 2 is shown.

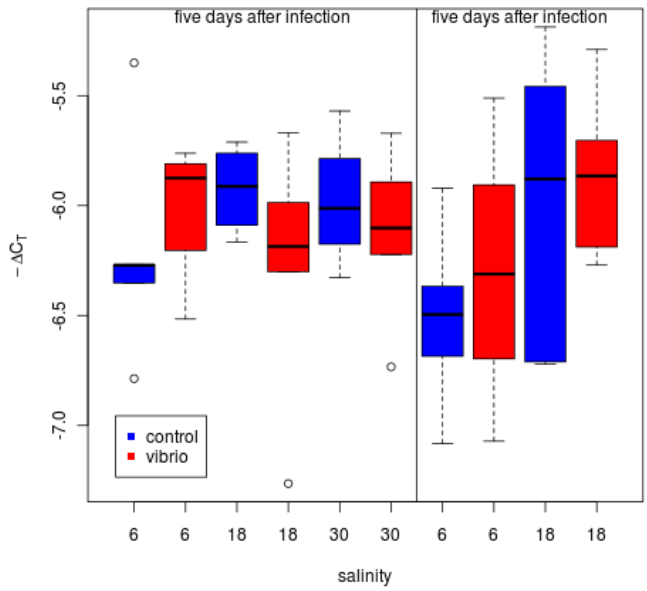


Figure 7.8: The relative activity ($-\Delta C_T$) of the immune gene kin one and five days after infection for Experiment 2 is shown.

Table 7.7: The degrees of freedom and t-values are shown for the results of the Pearson correlation of the single genes with the different immune parameters of Experiment 1.

t-value	df	***head kidney***			***blood***			
		lympho-cytes	**mono-cytes**	**G2M**	**lympho-cytes**	**mono-cytes**	**G2M**	**luminescence**
C3	24	0.875	-1.5916	0.282	**2.6978**	**-2.2077**	-0.6936	-0.401
cf	20	-0.6218	0.753	**2.3416**	-0.1769	0.5619	0.2295	0.3919
grcsf	22	**-3.4549**	**3.9771**	**3.2072**	-1.3713	1.8775	0.9098	-1.8334
IL	25	**-2.6854**	**3.1884**	**3.2368**	-1.3007	1.9944	0.6636	-2.0031
LA	19	0.2545	-0.3607	0.7575	1.0034	-0.7167	0.7065	-0.2093
TLR	25	-1.8523	1.04	1.8384	0.4572	0.1811	-0.0209	-0.6377
tnf	25	-1.0214	0.6027	0.3505	-0.1406	0.7144	-0.4406	-0.4891

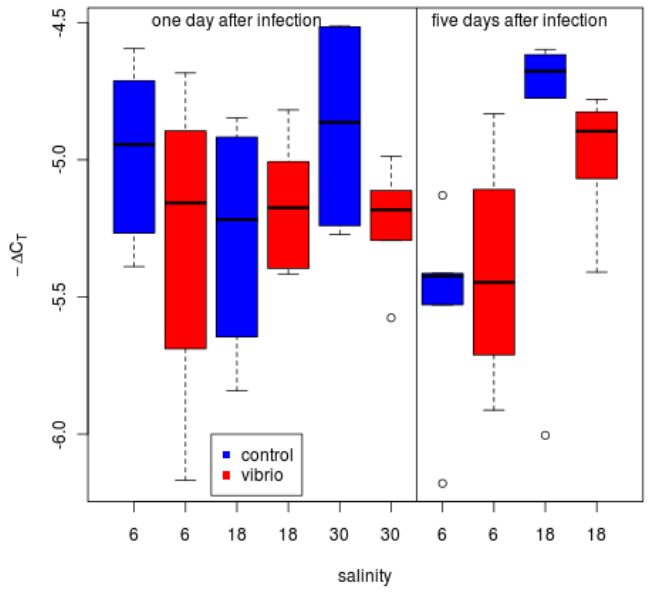


Figure 7.9: The relative activity ($-\Delta C_T$) of the immune gene LA one and five days after infection for Experiment 2 is shown.

Table 7.8: The degrees of freedom and F values for the results of the MANOVA for the gene expression of fish from 6, 18 and 30 PSU for the data from timepoint 1 of Experiment 2 are shown.

gene	**salinity** ***F***	**vibrio** ***F***	**salinity*vibrio** ***F***
over all	**3.3225**	1.1328	1.4698
C3	**4.8942**	1.9111	0.1281
cf	2.3653	0.4763	0.7137
grcsf	2.0124	0.0097	0.2535
hsp	0.5113	0.1982	**5.5624**
IL	0.1221	0.1564	0.9730
kin	0.0004	0.1111	0.9370
LA	0.1446	0.7608	0.7429
nramp	0.4706	0.0214	**5.0403**
TLR	0.7693	0.8517	1.0743
tnf	**3.7716**	0.9039	0.0251
df	2	1	2
Residuals	20		

Table 7.9: The degrees of freedom and F values for the results of the MANOVA for the gene expression of fish from 6 and 18 PSU including data from both timepoints of dissection of Experiment 2 are shown.

gene	**salinity** ***F***	**vibrio** ***F***	**timepoint** ***F***	**salinity*vibrio** ***F***	**salinity*timepoint** ***F***	**vibrio*timepoint** ***F***	**salinity*vibrio*timepoint** ***F***
over all	**3.7026**	0.4086	**9.9980**	0.5842	2.0815	1.9533	0.9527
C3	0.7080	0.0942	**15.1789**	0.0008	0.0143	2.2145	0.0532
cf	0.1897	1.3102	0.1053	0.5328	**4.8991**	1.3087	0.0361
grcsf	**5.0953**	0.1275	**13.2685**	0.0992	0.0797	0.0043	0.2696
hsp	0.0057	0.0225	**7.6338**	0.7882	0.3821	3.3564	2.1446
IL	0.0686	0.7286	**13.4360**	2.377E-05	0.4425	0.0002	1.1403
kin	2.1620	0.0610	0.0702	0.8807	2.1190	0.5626	0.4906
LA	2.2912	0.0782	0.1148	0.1196	**4.5876**	0.1715	1.1559
nramp	0.0585	0.0537	1.1402	0.0067	0.4347	3.3467	0.5799
TLR	0.5006	1.542E-05	**57.2491**	0.3361	2.0398	**7.2832**	0.1682
tnf	1.8300	0.9311	2.3359	1.0300	0.0659	0.0002	1.2237
df	1	1	1	1	1	1	1
Residuals	29						